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CONTENTS

ABSTRACT

Attempts have been made to study the involvement of Lys 41 (an active site residue) in the catalytic action of the enzyme ribonuclease A through nuclear magnetic resonance spectroscopy. The abnormal titration characteristics of this residue (low pK) together with experiments involving pH titration series, spin decoupling, the effect of inhibitors, the mapping of the cationic active site through the use of chromicyanide anion $[Cr(CN)_6]^{3-}$, and various chemical modification studies have been utilized to achieve this objective. The assignment of some new resonances of ribonuclease A has been accomplished through these studies.

In further efforts to study the three-dimensional structure of ribonuclease A attempts have been made to prepare and introduce strong binding sites for lanthanide cations at the N-terminus of the protein molecule. Both shift reagents (e.g. Yb^{3+}) as well as relaxation reagents (e.g. Gd^{3+}) have been investigated.

Calcium dipicolinate is known to impart thermal stability to biomolecules such as proteins. Since both Ca^{2+} and dipicolinic acid are major constituents of bacterial spores a possible role for the calcium salt of dipicolinic acid in the heat stabilisation of such spores is discussed. Weak complexation between the calcium in calcium dipicolinate and some spore constituents including amino acids and protein has been detected by both ^{1}H and ^{13}C NMR. Comparative studies have also been attempted between the involvement of Ca^{2+}, dipicolinic acid and calcium dipicolinate in the above mentioned process.

ABBREVIATIONS

Ca-DPA	Calcium salt of 2,6-pyridinedicarboxylic acid, Ca-DPA chelate (mono chelate)
DPA	Dipicolinic acid, 2,6-pyridinedicarboxylic acid
4-IDPA	4-Isothiocyanato-2,6-pyridinedicarboxylic acid
4-IPA	4-Isothiocyanatophthalic acid
ppm	Parts per million
RNase A	Ribonuclease A
RNase S	Ribonuclease S

CHAPTER 1

SOME GENERAL ASPECTS OF THE APPLICATION OF NMR IN BIOCHEMISTRY

Nuclear magnetic resonance (NMR) spectroscopy has been applied in the study of structural and dynamical aspects of biochemical systems. Such applications are relatively straight forward for small molecules (<1000 Daltons) but for macromolecules (<15000 Daltons) only limited success has been achieved. As far as the structural aspects are concerned, X-ray studies have been by far the most informative. However, the structure-function relationship of the molecules can often be better understood by NMR, the non-destructive technique that has the additional advantage of being used for solutions.

The immediate difficulties encountered in studying the NMR spectra of biomolecular systems are problems associated with sensitivity and resolution. In general proton NMR spectra of biomolecules like proteins are broad indecipherable envelopes. Sensitivity in these spectra is enhanced in studies at higher magnetic fields. Owing to the ubiquity of the hydrogen nucleus in biological systems higher magnetic fields do not always provide enhanced resolution. However the apparent resolution in the ^{1}H NMR spectra of biological systems may be improved by using a variety of resolution enhancement techniques. These techniques are completely artificial processes and their indiscriminate use may well result in detrimental broadening in the spectrum and/or loss of signals. This is especially true in cases where the spectra contain signals of low intensity. The methods used to obtain resolution enhanced spectra involve the manipulation of the

acquired free induction decay (FID) data [Lindon and Ferrige 1981]. The FID is multiplied by a function which increases the intensity at the latter end of the FID at the expense of the initial part. The multiplication causes a selective enhancement of some linewidths at the expense of others and this property can be utilised for baseline flattening. There is a decrease in the observed linewidth with a corresponding decrease in the signal-to-noise ratio. All functions aim to improve the resolution with a minimum loss in signal-to-noise ratio and with minor signal distortions.

There are various methods available for obtaining resolution enhanced spectra. The resolution of a complex NMR spectrum can be improved by subtracting a broadened spectrum from the original one. The broadening may either be brought about by multiplication of a free induction decay by an exponentially decaying function (convolution) or brought about by specific binding of a paramagnetic species with a long electron relaxation time [Campbell *et al.* 1973a]. The spectrum arising from the convolution method is referred to as the convolution difference spectrum. Paramagnetic ions affect the early part of the FID leading to an enlarged FID which in turn give rise to broad signals. Multiplication of the FID by a trapezoidal function is an improvement on using the simple exponential function since it reduces the beginning of the FID but leaves the latter part relatively unaffected. Overall this is similar to the convolution difference method except that more lineshape distortions are introduced. The sinebell method involves multiplying the FID by a sinewave of zero phase and period of twice the acquisition time. The sinebell function affects both the beginning and end of the FID equally but usually the decay vanishes into the noise well before the end of the acquisition

time and hence the convolution has the result of affecting the initial part of the FID most. A simple exponential multiplication broaden lines with an increase in signal-to-noise ratio. However trapezoidal multiplication, sinebell and convolution difference methods give rise to narrow lines and decrease in signal-to-noise ratio. Further resolution enhancement is effected by zero filling, in terms of data points [Lindon and Ferrige 1981].

In studies using paramagnetic ions (like Gd^{3+}, $[Cr(CN)_6]^{3-}$) it is imperative that a specific method of resolution enhancement be used throughout a complete biomolecule-paramagnetic ion titration study. The indiscriminate use of the resolution enhancement methods in a non-specific manner may well lead to unreliable results.

For a macromolecule like a protein, once a sensitivity and resolution enhanced spectrum is obtained, assignment and interpretation of the resonance can give information about its tertiary structure [Dwek 1973]. Each resonance can be defined by three parameters namely a chemical shift value, a coupling constant and a linewidth. While all resonances have a chemical shift value, some of them do not exhibit coupling phenomena (either because they are not coupled and/or because of line broadening in macromolecules due to longer correlation times). The chemical shift value arising from a particular nucleus in the spectrum of a biological macromolecule arises from

(i) the intrinsic chemical shift value of that nucleus,

(ii) the contribution from the anisotropy arising from the three-dimensional structure, and

(iii) the microenvironment of that nucleus.

For example, the ^1H NMR spectrum of ribonuclease A at a particular pH exhibited four distinct signals attributable to the C_2-H resonances

of the histidine residues present in the molecule [King and Bradbury 1971]. The coupling constants are the result of interaction between the spins of various nuclei in a residue. While some coupling constants often assist in assigning the resonances to particular nuclei, others provide more information about the structure of the molecule, especially the details of motions of various groups in a molecule in solution and the relative geometry of the interacting nuclei.

The assignment of the resonances to particular residues is necessary before the above mentioned information on structure and dynamics of the biomolecules in solution can be interpreted. This objective is achieved by several means. For instance, the effects of temperature, pH and ligands on the observed NMR spectrum can be very helpful, although the detailed interpretation of the results of such experiments usually relies heavily on the known crystal structure. Once the resonances are assigned, these above mentioned experiments may help in understanding some of the biological processes, for example, the catalytic action of the enzyme ribonuclease A [Roberts *et al.* 1969].

Various NMR techniques are also of great help in assigning the resonances in the spectra of the macromolecules. For example, selective spin decoupling experiments have been of considerable help in the matter (e.g. assignment of tyrosine ^1H resonances in ribonuclease A, Lenstra *et al.* [1979]). In recent times, the photo-CIDNP (Chemically Induced Dynamic Nuclear Polarisation) technique has been successful in assigning some of the aromatic proton resonances of histidine, tyrosine and tryptophan residues in protein molecules [Bolscher *et al.* 1979].

The use of chemical modification and isotopic enrichment have been extensively employed to study the problems regarding

assignment and interpretation. The applications of these chemical methods in the NMR study of biomolecules is becoming increasingly important and extensive literature is now available on this subject [Dwek 1973].

NMR relaxation processes are very sensitive to variation in molecular environment. Measurements of spin-lattice relaxation time (T_1), spin-spin relaxation time (T_2) and nuclear Overhauser effect (nOe) either directly or indirectly can provide knowledge about one or more of the following

(1) accessibility of the binding site on a macromolecule to various molecules, ions or solvent,

(2) mobility at the binding site,

(3) exchange kinetics,

(4) identification of the site of binding on both the small molecule and macromolecule, and

(5) role of the interaction in biological processes.

Chemical exchange effects on linewidths and relaxation times of small molecules can provide useful information about the kinetics of complex formation. Several attempts have been made to interpret the observed NMR parameters (linewidths and relaxation times) in terms of specific rotational and translational motions has met with some success particularly in membrane systems [James 1975].

The use of other nuclei in NMR, such as ^{13}C, ^{19}F, ^{31}P and ^{113}Cd and the quadrupolar nuclei like ^{2}H and ^{14}N have provided valuable information about some specific problems which otherwise are difficult to obtain from ^{1}H NMR alone [Dwek 1973].

Recently, a new concept named two-dimensional J-resolved ^{1}H NMR spectroscopy has been developed to greatly enhance the resolution in the ^{1}H NMR spectra of biopolymers and to facilitate assignment

of individual spectral components [Aue *et al.* 1976]. This technique has been most successfully applied to the protein, basic pancreatic trypsin inhibitor, especially in studying the aliphatic (upfield) portion of the spectrum [Nagayama *et al.* 1977].

The application of lanthanides (both shift and relaxation reagents) in the NMR study of biomolecules has been widespread and productive [Dwek 1973; Wüthrich 1976]. Large shifts are observed in the resonances of nuclei in paramagnetic complexes, compared with their corresponding positions in diamagnetic complexes. These shifts result from the interaction of the nuclei with the unpaired electron of the paramagnetic ion either by contact (Fermi or isotropic) or by pseudo-contact shifts.

The contact shifts occur due to delocalisation of the unpaired electron spin density at the resonating nucleus, transmitted through chemical bonds although other mechanisms such as hyperconjugation play a contributing role in some cases. The shift, $\Delta\nu_c$, for the lanthanides is given by [Lewis *et al.* 1962; Reuban and Fiat 1969]

$$\frac{\Delta\nu_c}{\nu_I} = -(2\pi\beta AJ(J+1)/3KT\gamma_I)(g_L(g_L-1)) \qquad 1.1$$

where A is the scalar coupling constant characteristic of a nucleus, J is the electronic spin angular momentum, β is the Bohr magneton, γ_I is the magnetogyric ratio of the nucleus (Hydrogen in this case), g_L is the Lande g factor, T is the absolute temperature and ν_I is the Larmor precession frequency.

Pseudo-contact or dipolar shifts are observed only if the magnetic field produced by the unpaired electron does not average to zero (i.e. anisotropic), such as the case with paramagnetic lanthanide complexes (excluding Gd^{3+}), first described in detail by McConnell

and Robertson [1958]. The pseudo-contact shift at a nucleus, $\Delta\nu_p$, is given by

$$\Delta\nu_p = K'(3\cos^2\theta-1)r^{-3} - K''(\sin^2\theta.\cos 2\phi)r^{-3} \qquad 1.2$$

where K' and K'' are ligand field parameters and r,θ and ϕ are the spherical coordinates of the nucleus relative to the lanthanide at the origin of the coordinate system. θ defines the angle between the principal axis Z and the vector joining the metal and the nucleus, and ϕ is the angular position in the plane perpendicular to the metal symmetry axis. Due to the existence of many possible conformations of the molecule in solution, the values of the parameters θ and r will be the result of averaging of the motions of the molecule, which means that the tumbling time (correlation time) is longer than the electron relaxation time. This happens so in the case of a nucleus in a lanthanide complex, where, the parameters corresponding to the axial symmetry alone are operative [Reuban and Fiat 1969]. The above mentioned equation 1.2 reduces to the form 1.3 in the case of axial symmetry, since K'' is zero due to cos 2ϕ being effectively reduced to zero by a dynamic averaging in solution

$$\Delta\nu_p = K'(3\cos^2\theta-1)r^{-3} \qquad 1.3$$

where $K' = -(\beta^2 J(J+1)\nu_I/45KT)(3g_{\parallel} + 4g_{\perp})(g_{\parallel} - g_{\perp})$

$g_{\parallel}$ and $g_{\perp}$ are the parallel and the perpendicular components of the electronic g-tensor with respect to the principal axis.

Hence, it is evident from the above mentioned relations that the pseudo-contact shifts are angular dependent and independent of the nucleus. Due to the peripheral position of the protons in

substrate molecules, the relative contribution of contact shifts may be very small and the observed shifts may be almost entirely due to the pseudo-contact shifts.

The nuclear relaxation in a dilute solution containing a paramagnetic solute, is often entirely dominated by pair-wise interactions between an unpaired electron spin, S, and the spin of the nucleus, I. These interactions cause considerable reduction in nuclear relaxation times, T_1 and T_2. In the case of nuclei bound near a paramagnetic site, the relaxation times are well represented by the Solomon [1955] - Bloembergen [1957] equations. Assuming $\nu_S >> \nu_I$ (the elctronic and nuclear larmor precession frequencies respectively), these are

$$\frac{1}{T_{1,M}} = \frac{1}{15}\,\frac{\gamma_I^2 g^2 S(S+1)\beta^2}{r^6}\left(\frac{3\tau_c}{1+\nu_I^2\tau_c^2}+\frac{7\tau_c}{1+\nu_S^2\tau_c^2}\right)$$

$$+\frac{2}{3}\,S(S+1)\left(\frac{A}{\hbar}\right)^2\left(\frac{\tau_e}{1+\nu_S^2\tau_e^2}\right) \qquad 1.4$$

and

$$\frac{1}{T_{2,M}} = \frac{1}{15}\,\frac{\gamma_I^2 g^2 S(S+1)\beta^2}{r^6}\left(4\tau_c+\frac{3\tau_c}{1+\nu_I^2\tau_c^2}+\frac{13\tau_c}{1+\nu_S^2\tau_c^2}\right)$$

$$+\frac{1}{3}\,S(S+1)\left(\frac{A}{\hbar}\right)^2\left(\frac{\tau_e}{1+\nu_S^2\tau_e^2}+\tau_e\right) \qquad 1.5$$

In both equations, the first terms arise from dipole-dipole interaction between the electron, S, and the nuclear spin, I, which is characterized by a correlation time, τ_c, which modulates this interaction. The modulation of the scalar interaction (often called isotropic nuclear-electron spin exchange interaction) give rise to the

second terms, which is characterized by a correlation time τ_e. S is the total electron spin, r is the distance between the nucleus and the paramagnetic ion and $A/\hbar$ is the electron-nuclear hyperfine coupling constant in Hz.

In both these equations, 1.4 and 1.5, the first term is predominant and so the relaxation times (or rates) are inversely proportional to r^6. Unlike the pseudo-contact shifts, these are not angular dependent. Hence, because of their desirable properties, both the shift reagents and the relaxation reagents have found widespread use in biological NMR.

Thus the methods and techniques for applying NMR spectroscopy to problems associated with biological macromolecules and biological processes have been summarized above. The major problem dealt with in this thesis involves the application of NMR spectroscopy to the study of ribonuclease A. Many of the techniques outlined above are utilized in an investigation of the structure and function of the enzyme, with particular reference to its catalytic action and the role of Lys 41, an active site residue. Further, the chemical modification of ribonuclease A was examined in an effort to introduce strong binding sites for lanthanides and hence probe the structure of the molecule in the immediate vicinity of such a site. NMR spectroscopy has also been used to study the binding of amino acids and ribonuclease A with the Ca-DPA complex. Such studies are a necessary prerequisite for the understanding of the induced thermal stabilisation of some biomolecules by Ca-DPA.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Ribonuclease A (Worthington Biochemical Corporation, phosphate free, lyophilized powder) was used for all the NMR work involving the direct use of the enzyme. Sigma ribonuclease A (Type IIA) was employed for all the preparatory purpóses.

Peptides and amino acids used were of high purity grade and obtained from BDH Chemicals Ltd, Fox Chemical Company, Vega-Fox Biochemicals, Nutritional Biochemicals Corporation, Fluka AG and Sigma Chemical Company. L-amino acids (and some DL-amino acids) were used throughout the study.

Lanthanide salts were purchased as the hydrated nitrates with a stated purity of 99% (Koch Light Laboratories and Alfa Products) and used without further purification.

Deuterium oxide (^2H_2O, 99.7%) was purchased from the Australian Atomic Energy Commission.

Other compounds were obtained from the following commercial sources:

May and Baker

Potassium ferricyanide and calcium nitrate (tetrahydrate).

Aldrich Chemical Company, Inc.

Dipicolinic acid.

Sigma Chemical Company

Uridine 3'-monophosphate (3'-UMP).

2.2 PREPARATION OF SOLUTIONS

Native and modified ribonuclease A solutions in the concentration range 1.45 mM - 7.25 mM were prepared in ^2H_2O for ^1H NMR. For ^{13}C NMR* 14.5 mM solutions of the enzyme in ^2H_2O were used.

All the labile protons in the protein molecule were exchanged for deuterium by keeping the protein solution (in ^2H_2O) at pH 5.0-6.0, in a water bath maintained at 55°C - 60°C for 10-15 minutes. While most of these solutions were used for obtaining the spectra directly, in some cases the H^2HO concentration in the sample were reduced by lyophilizing these solutions at least once. In others, the lyophilization from ^2H_2O was repeated two or three times. Constant ionic strengths were maintained in all these solutions by addition of 0.1 - 0.3 M NaCl or alternatively by 0.1 M KCl. All the solutions prepared in Chapter 5 contained 0.1 M KCl.

A 0.0172 M solution of Ca-DPA in ^2H_2O was used for both ^1H as well as ^{13}C NMR, because of the low solubility of Ca-DPA. Equimolar amounts of Ca-DPA and amino acid solutions in ^2H_2O were mixed and used to obtain the NMR spectra. The disodium salt of dipicolinic acid was prepared by carefully neutralising an aqueous solution of dipicolinic acid with a solution containing the appropriate amount of sodium bicarbonate and evaporating the solution to dryness. 5% solutions of amino acids in ^2H_2O were used for obtaining the ^{13}C spectra of the amino acids themselves.

pH titration series involving small molecules like amino acids were carried out with stock solutions of them in ^2H_2O. 0.5 ml of this solution was used for obtaining each spectrum at a particular pH.

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* All the ^{13}C NMR work described in this thesis refer to the natural abundance carbon-13 experiments.

2.3 MEASUREMENTS OF pH

The pH of the solutions used for NMR studies were measured using a Beckman model 4500 digital pH meter fitted with an Ingold combination electrode (6031-01). This enabled the pH of a solution to be measured in the NMR tube. Deuterium chloride was purchased from Aldrich Chemical Company, Inc. as a 20% solution in ^2H_2O with an isotopic purity of 99%. NaO^2H solutions were prepared by reacting sodium metal with ^2H_2O. 0.1 M solutions of ^2HCl and NaO^2H were used for adjusting the pH of the solution. The pH of each solution was measured before and after the NMR run and the average of these two was taken as pH of the solution.

An Orion digital pH meter (model 701A) fitted with a Beckman combination electrode was used for measuring the pH of the solutions other than those used for NMR.

The pH values of ^2H_2O solutions quoted in this thesis are pH meter readings uncorrected for the deuterium isotope effects.

2.4 NMR SPECTROSCOPY

A Jeol 100 MHz instrument (Model JNM-MH-100) located at the Chemistry Department (ANU) was used for obtaining ^1H NMR spectra at 100 MHz. This instrument operated on a continuous wave mode at an ambient temperature of 26°C. 0.1 M solutions were used for obtaining the spectra.

For dilute samples, a Varian CFT-20 instrument (pulsed Fourier transform spectrometer) operating at 79.6 MHz for ^1H was used. This instrument was also located at the Chemistry Department. Deuterium was used as the internal field-frequency lock. The probe temperature

was maintained at 30°C. Sodium 2,2',3,3'-tetradeutero-3-trimethylsilylpropionate (TSP) in a capillary tube was used as an external reference for the determination of chemical shifts of all resonances. Typically 500-1000 scans were accumulated for each sample. For proteins, 1000-4000 scans were accumulated. To obtain good spectral resolution, the free induction decay (FID) collected for each sample was Fourier transformed over twice the number of data points. A line-broadening factor of 1 Hz was employed for multiplying the FID. Convolution difference spectroscopy was also performed for some protein spectra to obtain resolution enhanced spectra [Campbell *et al.* 1973a]. In the spectra of protein samples H^2HO signal was suppressed by irradiating it with a pulse of considerable power. Variable temperature experiments involving proteins were also performed with this instrument.

Most of the ^1H NMR work involving the enzyme ribonuclease A and its derivatives were carried out on a Brüker HFX-270 spectrometer fitted with an Oxford Instruments magnet having a probe temperature of 20°C and located at the National NMR Centre. Again deuterium was used as an internal field-frequency lock. TSP in a capillary tube was used both for referencing all the resonances as well as for performing difference spectroscopy. Depending on the concentrations of the samples, 1000-4000 scans were accumulated to get a better signal-to-noise ratio. Resolution enhancement was achieved by the convolution difference spectroscopy or the double exponential multiplication (DM) or the trapezoidal multiplication (TM) or the sine-bell method of multiplication of the FID [Lindon and Ferrige 1980] using Nicolet 1180 computer and accompanying software. The following table illustrates the resolution enhancement method employed to obtain the spectra presented in the figures (Table 2.1). Difference spectroscopy was attempted with these spectra [King and Bradbury 1971] to confirm the changes observed in the shifts and the

TABLE 2.1

Figures	Resolution Enhancement Method
3.2, 3.3 and 3.12	Convolution difference spectroscopy (3Hz - 1Hz)
3.5, 3.25, 3.27, 3.30 and 4.4.	Double exponential multiplication
3.9, 3.17, 3.18, 3.19, 3.22 and 3.28	Trapezoidal multiplication*
4.1, 4.3, 4.7, 4.8, 5.13, 5,14 and 5.15	Sine-bell

* T_1 = 20, T_2 = 2000 over 4K data points, where T_1 and T_2 define the branch points in the trapezoidal multiplication.

broadening. The H^2HO signal in the protein spectrum was suppressed by irradiating it with a homogated pulse of duration 0.2 s prior to the 90° one. In the spin decoupling experiments the H^2HO signal was not irradiated since there was no facility to irradiate the H^2HO signal and the region of interest simultaneously.

The ^{13}C spectra of the amino acids and dipicolinic acid (disodium salt) were obtained on the Varian CFT-20 instrument operating at 19.9 MHz for carbon-13. Proton noise decoupled (^{13}C-{^1H}) spectra were obtained that gave fully decoupled spectra with the nOe. At least 20000 scans were accumulated to get a reasonable spectrum and a 2 Hz line-broadening factor was employed. A small amount of dioxane was used as an internal standard and a spectral width covering 0-250 ppm was employed. A 45° pulse was used with a recycle time of 1.8 s. Since the decoupler was on all the time, the temperature of the sample was always higher than the normal probe temperature, irrespective of the instrument employed.

The ^{13}C spectra of Ca-DPA-amino acid chelates were obtained on a Jeol FX-90Q instrument (operating at 22.5 MHz for Carbon-13)

located at the John Curtin School of Medical Research, ANU. This instrument also operated at a probe temperature of 30°C and the samples were locked externally. All the other parameters were the same as mentioned above.

The ^{13}C spectra of ribonuclease A was obtained on the Brüker HFX-270 spectrometer operating at 67.5 MHz for carbon-13. The other parameters were the same as outlined above but no reference (internal or external) was used.

2.5 DETERMINATION OF DISSOCIATION CONSTANT

The apparent dissociation constant pK was determined from a plot of $\log[(\delta_A - \delta)/(\delta - \delta_B)]$ versus pH, where δ, δ_A and δ_B are the chemical shifts of the resonances of the sample in solution being measured, the sample in its acid form and base form respectively. The simple Henderson-Hasselbalchtreatment requires that this plot be linear with a gradient of unity and the pK is the value of the pH at which the log term is zero. The line of best fit was obtained by the method of least squares. The plots of the pH titration curves and the determination of apparent pK values were obtained from a computer programme written by M.D. Ridgway and later refined by J. Reimers.

2.6 OTHER EXPERIMENTAL METHODS

Mass spectra were recorded on a Varian CH 7 spectrometer and infrared spectra on a Perkin-Elmer 683 infrared spectrometer (located at the Research School of Chemistry, ANU).

For the plots described in Chapter 3 (Figures 3.6, 3.20 and 3.21) and 5 (Figure 5.9), the line of best fit was obtained by the method of least squares.

2.7 ENZYMATIC ACTIVITY ANALYSES

The enzymatic activities of the native ribonuclease A and its derivatives toward ribonucleic acid (Sigma, torula yeast, Grade VI) were tested by the method of Kunitz [1946] and by the method of Kalnitsky *et al.* [1959]. In the Kunitz method the rate of decrease in absorbance of the ribonucleic acid at 300 nm is recorded to determine the enzymatic activity. The Kalnitsky's method makes use of the measurement of absorbance (at 260 nm) of the acid soluble oligonucleotides produced by the action of ribonuclease A and its derivative on yeast ribonucleic acid.

2.8 AMINO ACID ANALYSES

Samples for the amino acid analyses were hydrolyzed by heating in 6 M HCl for 22 hours at 110°C *in vacuo*. Analyses were carried out using a Beckman 120 B amino acid analyzer. The two column analysis procedure of Spackman *et al.* [1958] was employed. Thanks are due to Mr L.B. James of Biochemistry Department, John Curtin School of Medical Research, for carrying out all the amino acid analyses mentioned in this thesis.

CHAPTER 3

^{1}H NMR STUDIES OF LYSINE 41 OF RIBONUCLEASE A

3.1 INTRODUCTION

Bovine pancreatic ribonuclease A is one of the most thoroughly studied of all the enzymes. Its function is to digest RNA either in diet as is the case with most species or of the bacteria in the rumen (of the ruminants). This enzyme has a molecular weight of 13680 (anhydrous form) and contains 124 amino acid residues [Richards and Wyckoff 1971]. It contains more basic amino acid residues than acidic ones as evident from its isoelectric point (>9.0). It is also interesting to note that this protein does not contain any tryptophan residue in its polypeptide chain.

The x-ray structure of ribonuclease A is not yet clearly established. Information about the three-dimensional structure of this enzyme is available from the investigation by Wyckoff *et al.* [1967] on ribonuclease S and also from the work of Kartha *et al.* [1967] and Carlisle *et al.* [1974] on ribonuclease A. Ribonuclease S is derived from ribonuclease A by cleavage between residues 20 and 21 in the polypeptide chain. The single polypeptide chain of ribonuclease A twists forwards and backwards forming a U-shaped structure enclosing a cleft in which lie the residues responsible for the activity of the enzyme [Carlisle *et al.* 1974]. The three-dimensional structure is partially composed of an α-helix (*ca.* 19%) together with a large amount of β structure.

Uncertainty prevails over various aspects of the catalytic action of ribonuclease A despite extensive studies. The hydrolysis of ribonucleic acid by ribonuclease A is brought about in two stages [Richards and Wyckoff 1971]. The transphosphorylation stage [Usher *et al.* 1970] involves addition of the 2'-OH group (on the 3'-ribose sugar) to the phosphate group cleaving the ribonucleic acid chain at the 5'-end and thereby yielding a 2'-3' cyclic phosphate and a free 5'-OH group (Figure 3.1a). The hydrolysis involves addition of H_2O to the cyclic intermediate, yielding a terminal 3'-phosphate monoester [Brown *et al.* 1952; Markham and Smith 1952].

Several proposals have been made to explain the mechanistic action of ribonuclease A. From chemical, x-ray and nuclear magnetic resonance studies Roberts *et al.* [1969] presented evidence for the mechanism proposed originally by Findlay *et al.* [1962]. In the transphosphorylation stage this mechanism involves (1) the removal of the proton on the 2'-OH by a histidine in the base form, (2) the attack of the $2'\text{-}O^{\theta}$ on the phosphate group which also rotates towards it to form a trigonal-bipyramidal intermediate (TBP), and (3) protonation of the 5'-O by a histidine in the acid form which results in the breakdown of the TBP to form free 5'-ROH and a 2'-3' cyclic intermediate. The TBP formed has the $2'\text{-}O^{\theta}$ and 5'-O leaving group in apical positions and the addition of $2'\text{-}O^{\theta}$ to the phosphate group is by an in-line mechanism i.e. on SN_2 like reaction [Usher *et al.* 1972; Eckstein *et al.* 1972; Usher 1969]. The hydrolysis step is postulated to be exactly the reverse of the transphosphorylation step. Roberts *et al.* [1969] have also presented evidence against an adjacent mechanism requiring pseudo-rotation of the TBP (Figures 3.1a and 3.1b), previously proposed by Witzel [1963], Wang [1968] and Hammes [1968].

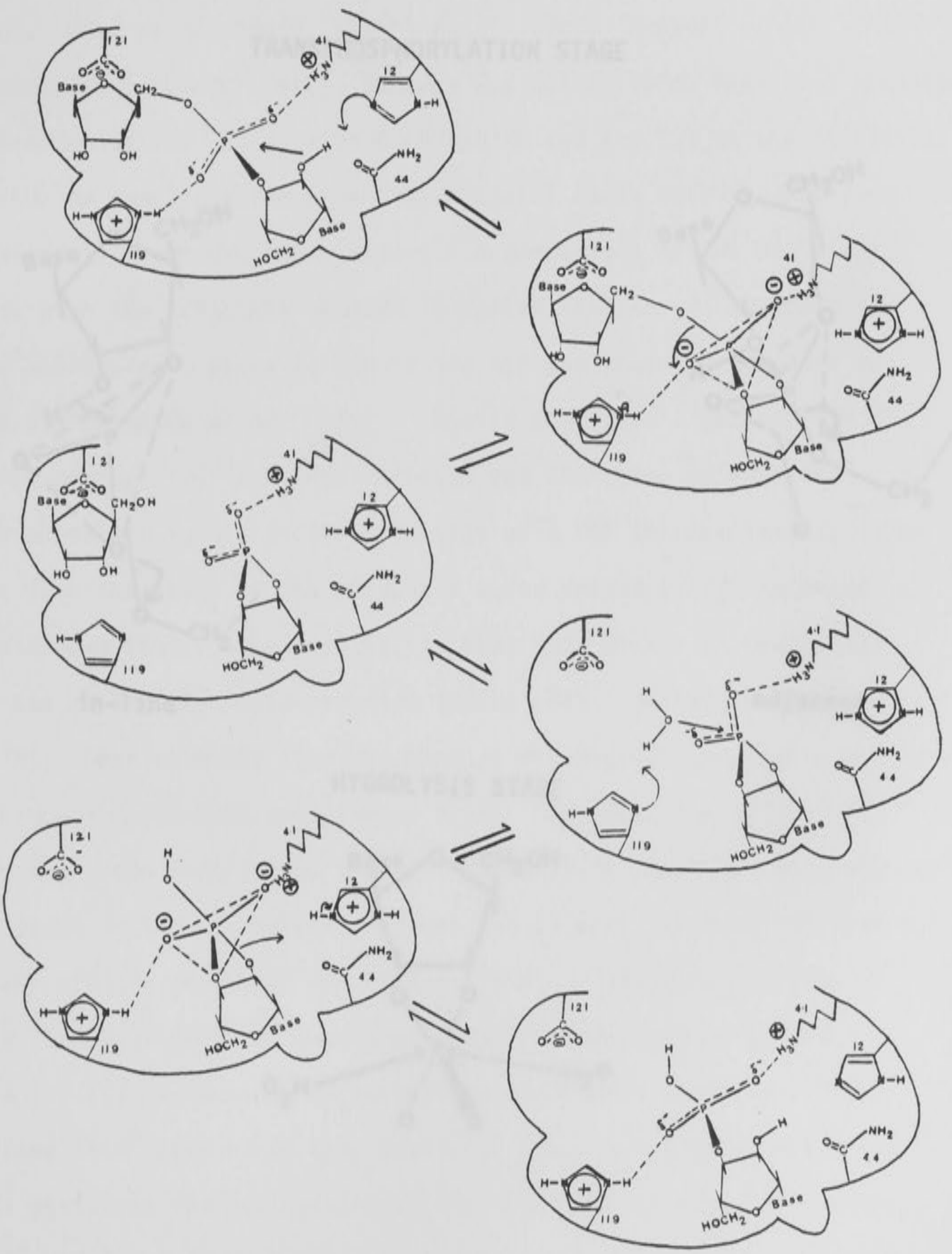
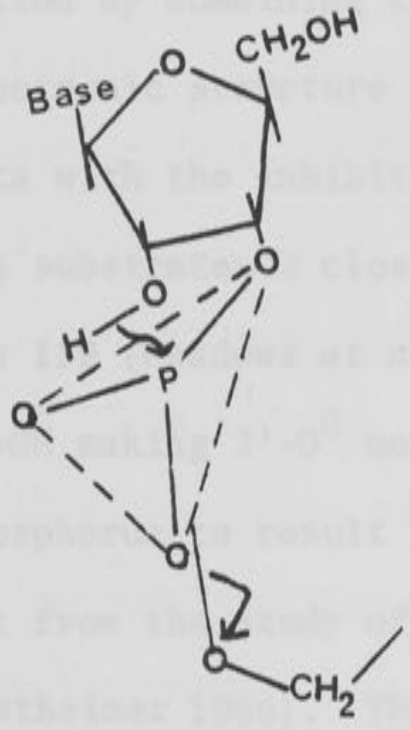


Figure 3.1a Catalytic action of ribonuclease A (based on the Figure from Roberts *et al.* [1969]).

TRANSPHOSPHORYLATION STAGE



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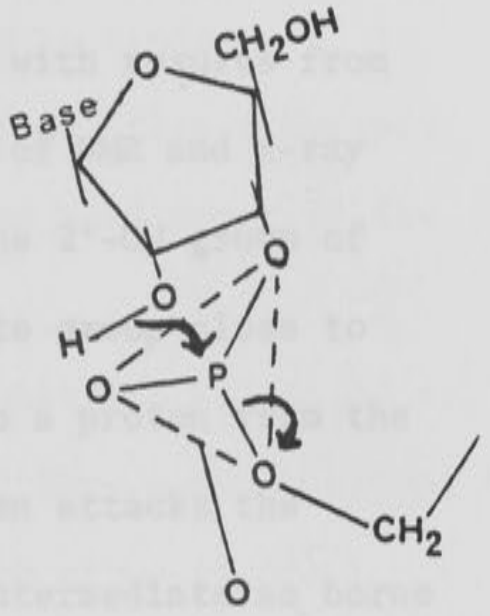

adjacent

HYDROLYSIS STAGE

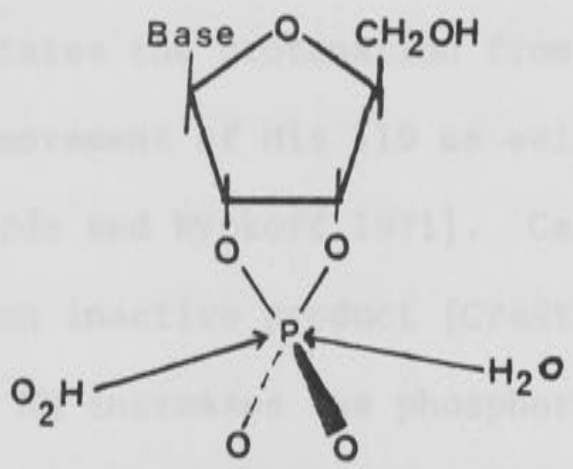

Figure 3.1b Catalytic action of ribonuclease A.

Chemical studies aided by x-ray and NMR investigations have suggested that histidine 12, histidine 119 and lysine 41 are involved in the catalytic action of the enzyme [Crestfield *et al.* 1963; Hirs *et al.* 1965; Kartha *et al.* 1967; Wyckoff *et al.* 1967; Meadows and Jardetzky 1968]. Deakyne and Allen [1979] have also assigned roles to Asn 44, the 119-120 backbone NH and Asp 121 in the catalytic action by combining the known experimental facts with results from electronic structure calculations. A comparison of NMR and x-ray data with the inhibitor studies indicated that the 2'-OH group of the substrate was close to His 12 and the phosphate group close to His 119 [Meadows *et al.* 1969]. His 12 abstracts a proton from the 2'-OH making 2'-$O^{\ominus}$ more nucleophilic and this then attacks the phosphorus to result in the formation of a TBP intermediate as borne out from the study of the phosphate ester hydrolysis [Dennis and Westheimer 1966]. The possibility that this proton is abstracted by Asn 44 has also been proposed [Gutte 1975; Deakyne and Allen 1979]. Even when His 12 was blocked or missing some enzymatic activity was reported [Machuga and Klapper 1975]. The position of the $RO^{\ominus}$ leaving group facilitates the protonation from His 119. This process is aided by a small movement of His 119 as well, as inferred from the x-ray studies [Richards and Wyckoff 1971]. Carboxymethylation of His 119 resulted in an inactive product [Crestfield *et al.* 1963]. The 119-120 backbone NH increases the phosphorus electrophilicity aiding 2'-$O^{\ominus}$ attack and also activates the leaving group while Asp 121 positions the purine ring of the substrate by means of a water-mediated hydrogen bond [Deakyne and Allen 1979].

Richards and Wyckoff [1971] have shown that Lys 41 is near His 119 and His 12 in the active site from the x-ray structure of

ribonuclease S. The proposed role of Lys 41 is to increase the phosphorus electrophilicity and stabilise the TBP by interacting with a phosphoryl oxygen [Richards and Wyckoff 1971; Roberts *et al.* 1969; Brown and Bradbury 1976]. Gutte [1977] prepared a 63 residue analogue of ribonuclease A, in which Lys 41 was replaced by tyrosine or glutamine, and was found to behave like ribonuclease A in its catalytic action (showed a low activity) and specificity in substrate binding. Although such results should be treated with caution, this experiment indicated that Lys 41 might function as a hydrogen bond donor in the catalytic action.

Ribonuclease A has ten lysine residues at positions 1,7,31,37,41, 61,66,91,98 and 104. The N-terminal lysine has a free α-amino group besides an ε-amino group and the pK of the α-amino group of Lys 1 in the native enzyme was found to be 7.6 at 20°C [Bradbury *et al.* 1978]. From the methylation studies, after correction for the effects of methylation, a pK of 6.6 was found for the α-amino group of the same residue, a pK of 8.6 for the ε-amino group of Lys 41 and pK values ranging from 10.6 to 11.2 for the other lysine ε-amino groups [Brown and Bradbury 1975]. Similar values were also reported from the ^{13}C NMR studies of [^{13}C]-methylated ribonuclease A [Jentoft *et al.* 1979]. From kinetic studies of the reaction between ribonuclease A and 1-fluoro-2,4-dinitrobenzene (FDNB), Murdock *et al.* [1966] deduced the pK for Lys 41 to be 8.8 ± 0.1. From similar studies with 4-sulfonyloxy-2-nitrofluorobenzene (SNFB), Carty and Hirs [1968] reported a pK value of 9.1 ± 0.1 for the same residue and indicated that pK values up to 9.5 could be inferred depending on the pH used. From the reactivity towards trinitrobenzenesulfonic acid (TNBS) Goldfarb *et al.* [1974] concluded that the pK of this residue was about 9.0. The abnormally low pK value of this residue has been

attributed to its presence in a cationic active site. The positively charged residues present in or near the active site as borne out from the x-ray studies are Lys 1, Lys 7, Lys 66, Arg 10, Arg 33, Arg 39, Arg 85, His 12 and His 119 besides Lys 41. The presence of these positively charged residues facilitates the dissociation of the proton from the positively charged ε-amino group of Lys 41 by repulsion. It is not known for certain whether Arg 39 and Arg 85 are the residues that are mainly responsible for the low pK of Lys 41 although a recent kinetic study tends to support this proposal [Iijima *et al.* 1977].

Chemical studies involving selective modification of Lys 41 by a dinitrophenyl group [Hirs 1962], sulfonyloxynitrophenyl group [Carty and Hirs 1968], carboxymethyl group [Heinrikson 1966], guanidination [Klee and Richards 1957] and methylation [Paik and Kim 1972; Means and Feeney 1968] all resulted in a product of very low activity. The methylated derivatives were almost inactive. Although a partial change in conformation has been proposed to account for the loss in activity of these derivatives of ribonuclease A [Ettinger and Hirs 1968], it is not known for certain whether this conformational distortion, steric effects and electronic effects either individually or collectively are responsible for the loss in activity. Allewell *et al.* [1973] found from the x-ray studies of ε-41 dinitrophenyl-ribonuclease S that dinitrophenylation displaced the ε-amino group of Lys 41 by 3 Å from the active site in addition to reducing the charge on this group. From the studies of the interaction between ribonuclease A and the ribonuclease inhibitor from human placenta it was reported that such interaction involved the positively charged ε-amino group of Lys 41 of ribonuclease A, and specific chemical modification of this group resulted in a reduced interaction between

the above mentioned species [Blackburn *et al.* 1979, 1980]. The presence of phosphate in the active site was also found to inhibit the reaction with Lys 41 [Hirs *et al.* 1965]. Further comparative sequence data compiled by Welling *et al.* [1973] have established Lys 7, -41 and -66 to be invariant in a large number of ribonuclease enzymes.

Although these experimental observations clearly implicate the involvement of Lys 41 as a part of the catalytic apparatus of ribonuclease A, definitive evidence is still lacking. Detailed information on the interactions of Lys 41 with active site ligands is not available from x-ray studies because the lysine side chains are not resolved on the crystalline enzyme. The current evidence on the role of lysine residues in the active site of ribonuclease A appears to contain a conflict in that one set of data strongly implicate lysine residues as part of the active site, while another set raises the possibility that they do not interact with substrate in the active site and only conformational changes are responsible for the loss in activity in the derivatives of ribonuclease A in which Lys 41 is modified. The present investigation was undertaken using NMR spectroscopy as a tool to seek further evidence for the role of Lys 41 in catalysis by ribonuclease A. In view of the anomalous pK of this residue one might anticipate abnormal NMR behaviour of the groups involved at the active site.

3.2 EXPERIMENTAL

3.2.1 Preparation of Ribonuclease S

Ribonuclease S was prepared by the method of Doscher [1967]. A 2% solution of ribonuclease A in 0.1 M Tris-HCl, pH 8.0, was mixed

thoroughly with a 1% solution of subtilopeptidase A in the same buffer, at a weight ratio of 250:1. The reaction mixture was left aside until a maximum amount of ribonuclease S was formed. As ribonuclease S is rapidly digested by trypsin while ribonuclease A is not, this property is used to estimate the amount of ribonuclease S formed. Aliquot samples (10 µl, 200 µg ribonuclease A) were removed at three hourly intervals and mixed with 500 µl of 0.01 M HCl. After one hour at room temperature a 50 µl sample of this solution was added to 10 µl of freshly prepared trypsin solution (0.1% in 0.5 M Tris-HCl, pH 8.0). A second 50 µl sample was added to 10 µl of 0.5 M Tris-HCl, pH 8.0 buffer. After one hour at room temperature 20 µl of each of these solutions (6.6 µg of ribonuclease A) were taken for measurement of the enzymatic activity by the Kunitz [1946] method. After ascertaining the maximum extent of ribonuclease S formation, the reaction mixture was acidified to pH 2.0 by dropwise addition of 1 M HCl which denatured the subtilopeptidase A. The solution was stirred at room temperature for one hour, cooled to 5°C and brought to pH 6.0 by dropwise addition of 5 N ammonium hydroxide. The solution was then dialyzed extensively against distilled water at 4°C for one to two days and then chromatographed.

About 100 mg of the substance was layered on the top of a 2 x 40 cm jacketted column through which cold water at 5°C was kept circulated. The column was packed with Bio-Rex 70 (200-400 mesh) ion-exchange resin (Bio Rad Laboratories) and was eluted with 0.2 M phosphate buffer, pH 6.47 at the rate of 15 ml/hr. This buffer was also used to equilibrate the column. Fractions of constant volumes were collected by using a LKB 3400 B Radirac automatic fraction collector. The effluent was monitored at 280 nm using a

LKB 8300 A Uvicord II Spectrophotometer and LKB 6520 chopper bar recorder. The fractions corresponding to ribonuclease S were determined by testing for its activity in the presence of trypsin as mentioned above. The pooled fractions were concentrated on a rotary evaporator (bath temperature <35°C) to a volume of 10 ml and dialyzed extensively against distilled water for several days to remove the bulk of the phosphate. The residual phosphate was removed by placing the substance on a 1 x 100 cm column of fine grade sephadex G-25 (Pharmacia) equilibrated with 5% acetic acid. The column was eluted with the same solution. The fractions containing the protein were pooled, dialyzed extensively to remove acetate and then lyophilized. Phosphate analysis by the microanalytical service, John Curtin School of Medical Research, indicated that there was less than 0.01% phosphorus present.

3.2.2 Preparation of Nona and Decaguanidino Ribonuclease A

The method of Glick and Barnard [1970] was used for the preparation of these compounds. The guanidination was achieved by treatment with 1-guanyl-3,5-dimethylpyrazole (GDMP) purchased from Eastman Organic Chemicals and recrystallised from hot water. 300 mg of ribonuclease A was dissolved in 5 ml of distilled water and pH adjusted to 10.0 with 1 M NaOH. 270 mg of GDMP was dissolved in 5 ml of distilled water and the pH adjusted to 10.0 with 6 M NaOH. The two solutions were mixed (pH 10.0) and stirred gently at room temperature. After 16 hours the pH was found to have decreased slightly, so GDMP (210 mg) dissolved in 2 ml of distilled water and with pH adjusted to 10.0 was added to the reaction mixture. After readjusting the pH of the total mixture to 10.0 the solution was kept for a

further 36 hours. The pH of the reaction mixture was then lowered to 6.0 with 1 M HCl and the solution was dialyzed extensively against distilled water and lyophilized.

Guanidination of ribonuclease A gives a mixture of the nona and decaguanidinated products. They were separated by a modification of the method of Glick and Barnard [1970]. Before proceeding with the bulk separation an analytical column was set up. Approximately 10 mg of the guanidinated sample was dissolved in *ca.* 0.5 ml of 0.15 M phosphate buffer (pH 6.6) and applied to a 0.6 x 40 cm column of Bio-Rex 70 (200-400 mesh) ion-exchange resin equilibrated with the same buffer. A linear gradient buffer for elution was set up with 70 ml each of 0.15 M phosphate buffer (pH 6.6) and 0.4 M phosphate buffer (pH 6.6) at the rate of 6 ml/hr. A 2.1 x 40 cm column was used for the bulk separation and approximately 300 mg of the product was applied and eluted as described above. One litre each of the above mentioned buffers were used in the construction of the linear gradient eluent and the elution rate was 42 ml/hr. 7 ml fractions were collected. The fractions containing the products were collected, monitored and recorded as mentioned in section 3.2.1. The bulk and the residual phosphate was removed as described in that section.

3.2.3 Preparation of Methylated Proteins

Proteins were methylated by the method of Means and Feeney [1968]. The protein was dissolved in 0.2 M borate buffer (pH 9.0) at a concentration of 5-10 mg/ml and cooled to 2°C. 0.7 mg/ml of potassium borohydride was added, followed by six 0.5 µl/ml additions of 37% formaldehyde at five minute intervals. For ribonuclease A the addition of potassium borohydride and formaldehyde was repeated to

increase the degree of methylation. The reaction mixture was lyophilized after being extensively dialyzed. Ribonuclease A and nonaguanidino ribonuclease A were methylated by this procedure.

3.2.4 Preparation of Potassium hexacyanochromate (III)

Potassium hexacyanochromate (III) ($K_3[Cr(CN)_6]$) was prepared by a modification [Chemistry Department, ANU] of the method of Bigelow [1946]. The reactions can be depicted as follows

$$
\begin{aligned}
Cr_2O_7^{2-} + 3SO_2 + 2H^+ &\rightarrow 2Cr^{3+} + 3SO_4^{2-} + H_2O \\
Cr^{3+} + 3OH^- &\rightarrow Cr(OH)_3 \\
Cr(OH)_3 + 3CH_3COOH &\rightarrow Cr(CH_3COO)_3 + 3H_2O \\
Cr(CH_3COO)_3 + 6KCN &\rightarrow K_3Cr(CN)_6 + 3CH_3COOK
\end{aligned}
\qquad 3.1
$$

A solution of potassium dichromate (25 g) in distilled water (500 ml) was reduced by passing sulfur dioxide into the solution. Excess sulfur dioxide was then removed by boiling. Aqueous ammonia was then added to the vigorously stirred boiling solution to precipitate the chromium (III) hydroxide. The addition of ammonia was stopped as soon as the faint odour of ammonia could be detected. The grey-green precipitate was filtered, washed with hot water and dissolved in glacial acetic acid (100 ml). The solution was then concentrated almost to dryness and then diluted with water (180 ml). A solution of potassium cyanide (75 g) in water (300 ml) was then added and the dark red solution was concentrated on a water bath. The impure product which separated was filtered and recrystallized from water (care must be taken to avoid prolonged heating since this caused some decomposition of the complex). Potassium hexacyanochromate (III) was obtained as pale yellow crystals (yield 36 g, 65%).

Found: K, 36.1%; Cr, 15.5%; C, 21.8%; N, 24.0%.

Calculated for $K_3Cr(CN)_6$: K, 36.0%; Cr, 16.0%; C, 22.0%; N, 26.0%.

3.3 RESULTS AND DISCUSSION

3.3.1 ^1H NMR Studies of Ribonuclease A and Ribonuclease S at Varying pH

In the NMR spectrum* of ribonuclease A, the ε-CH_2 protons of the lysine residues in the protonated form resonate around 3.0 ppm. As the pH is increased these resonances shift upfield due to an increase in shielding caused by the deprotonation of the ε-amino group. The ε-CH_2 protons appear as triplets coupled to the δ-CH_2 protons and their coupling constants are of the order of 7.7 Hz [Bundi and Wüthrich 1979]. Figure 3.2 shows the spectra obtained when these lysine residues were titrated in the present work. Because of the overlapping signals (the titration of at least nine ε-CH_2 triplets) it was difficult to get good titration curves for these residues. At higher pH as many as ten peaks seemed to titrate because of the difference in titration behaviour of these residues (the pK's of each of these residues differ slightly due to the difference in their microenvironment).

No group that titrated with a pK of about 9.0 corresponding to that of Lys 41 could be detected in the above series of titrations. Although ribonuclease A was shown to be less prone to aggregation [Richards and Wyckoff 1971] than many other enzymes, dilute solutions of the enzyme were used for these titration experiments at a temperature of 40°C. Even when the titration of ribonuclease A was performed

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* Unless otherwise stated, for all the work described in this thesis, spectra refers to the ^1H NMR spectra, titration to the pH titration and resonance to the proton resonance.

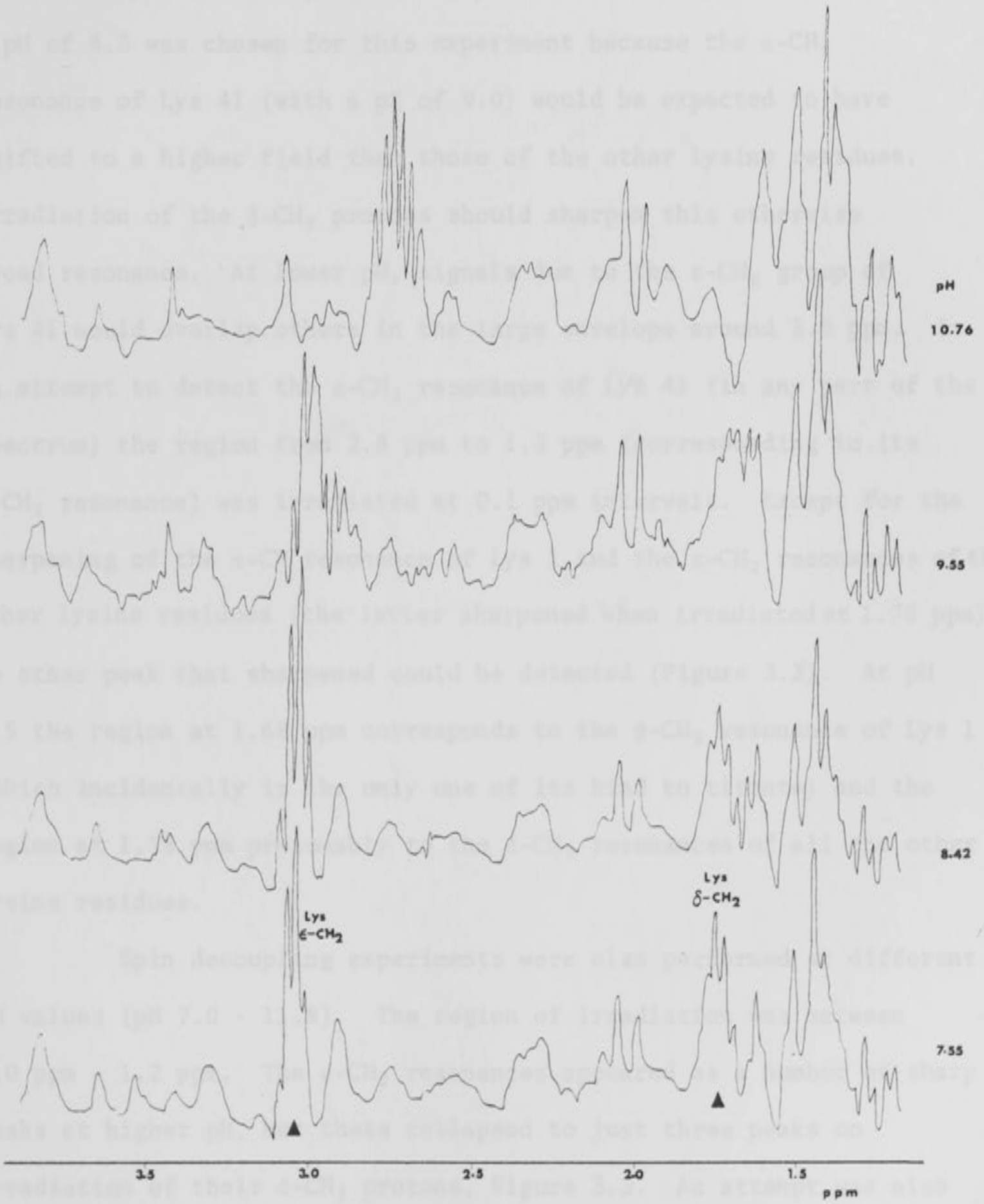

Figure 3.2 Titration of lysine residues of ribonuclease A. ^{1}H NMR spectra obtained at 270 MHz with 4.35 mM of ribonuclease A at 40°C.

at other concentrations and temperatures it was not possible to detect a lysine residue exhibiting a low pK.

In an alternative approach, spin decoupling experiments were performed by irradiating the δ-CH_2 protons of the lysine residues. A pH of 8.5 was chosen for this experiment because the ε-CH_2 resonance of Lys 41 (with a pK of 9.0) would be expected to have shifted to a higher field than those of the other lysine residues. Irradiation of the δ-CH_2 protons should sharpen this otherwise broad resonance. At lower pH, signals due to the ε-CH_2 group of Lys 41 would overlap others in the large envelope around 3.0 ppm. In an attempt to detect the ε-CH_2 resonance of Lys 41 (in any part of the spectrum) the region from 2.8 ppm to 1.3 ppm (corresponding to its δ-CH_2 resonance) was irradiated at 0.1 ppm intervals. Except for the sharpening of the α-CH resonance of Lys 1 and the ε-CH_2 resonances of the other lysine residues (the latter sharpened when irradiated at 1.78 ppm) no other peak that sharpened could be detected (Figure 3.3). At pH 8.5 the region at 1.68 ppm corresponds to the β-CH_2 resonance of Lys 1 (which incidentally is the only one of its kind to titrate) and the region at 1.78 ppm presumably to the δ-CH_2 resonances of all the other lysine residues.

Spin decoupling experiments were also performed at different pH values (pH 7.0 - 11.8). The region of irradiation was between 2.0 ppm - 1.2 ppm. The ε-CH_2 resonances appeared as a number of sharp peaks at higher pH, but these collapsed to just three peaks on irradiation of their δ-CH_2 protons, Figure 3.3. An attempt was also made to obtain good titration curves for the remaining lysine residues (at least nine) in the above mentioned manner but was unsuccessful because of the difficulties arising from irradiating all the lysine δ-CH_2 protons simultaneously. This was further complicated at higher pH.

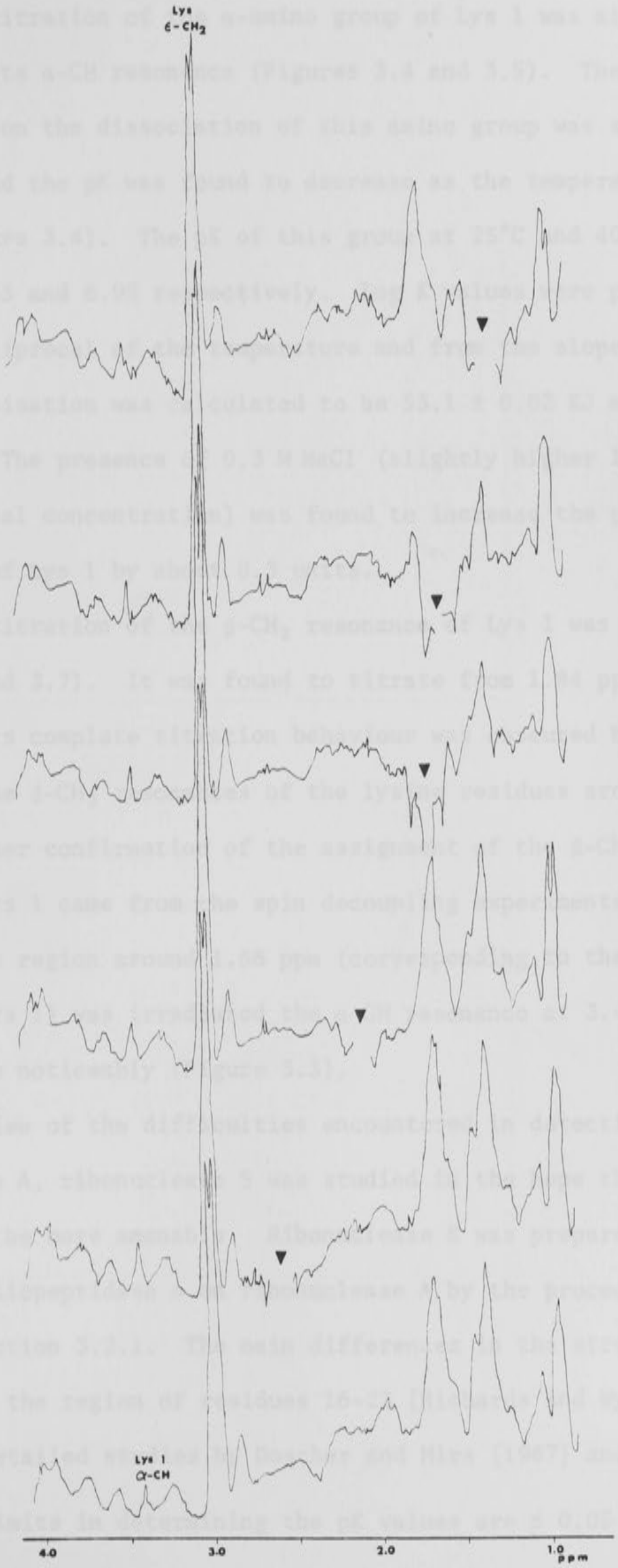

Figure 3.3 Spin decoupling experiments on ribonuclease A at 270 MHz. 7.25 mM of RNase A at pH 8.5 and 20°C was used. The region of irradiation is indicated by the mark ▼.

The titration of the α-amino group of Lys 1 was also observed by monitoring its α-CH resonance (Figures 3.4 and 3.5). The effect of temperature on the dissociation of this amino group was studied (Figure 3.4) and the pK was found to decrease as the temperature was increased (Figure 3.4). The pK of this group at 25°C and 40°C was found to be 7.35 and 6.95 respectively. Log K values were plotted against the reciprocal of the temperature and from the slope the enthalpy of ionisation was calculated to be 53.1 ± 0.02 KJ $mole^{-1}$* (Figure 3.6). The presence of 0.3 M NaCl (slightly higher level than the physiological concentration) was found to increase the pK of the α-amino group of Lys 1 by about 0.3 units.

The titration of the β-CH_2 resonance of Lys 1 was also noted (Figures 3.5 and 3.7). It was found to titrate from 1.84 ppm but the detection of its complete titration behaviour was obscured by the titration of the δ-CH_2 resonances of the lysine residues around 1.7 ppm. Further confirmation of the assignment of the β-CH_2 resonance of Lys 1 came from the spin decoupling experiments. At pH 8.5 when the region around 1.68 ppm (corresponding to the β-CH_2 resonance of Lys 1) was irradiated the α-CH resonance at 3.41 ppm sharpened quite noticeably (Figure 3.3).

In view of the difficulties encountered in detecting Lys 41 in ribonuclease A, ribonuclease S was studied in the hope that this molecule might be more amenable. Ribonuclease S was prepared by the action of subtilopeptidase A on ribonuclease A by the procedure outlined in section 3.2.1. The main differences in the structure of A and S are in the region of residues 16-23 [Richards and Wyckoff 1971]. More detailed studies by Doscher and Hirs [1967] and by Gross

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* The error limits in determining the pK values are ± 0.05 pK units.

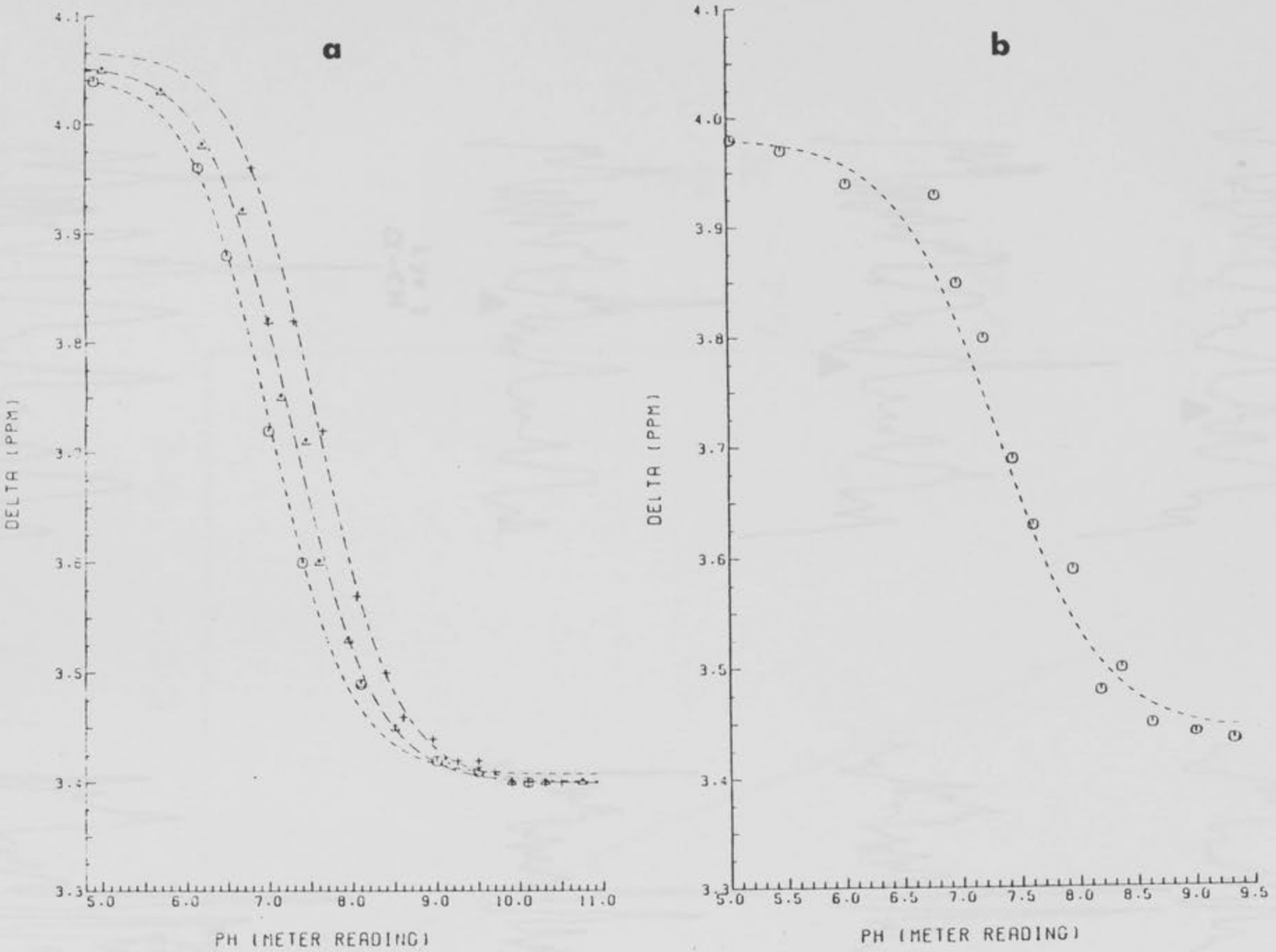


Figure 3.4 Titration curves for the α-CH resonance of Lys 1 at different temperatures.

(a) RNase A - 4.35 mM

+ - 20°C; Δ - 25°C; O - 40°C.

(b) RNase A-7.25 mM; NaCl - 0.3 M; Temperature - 35°C.

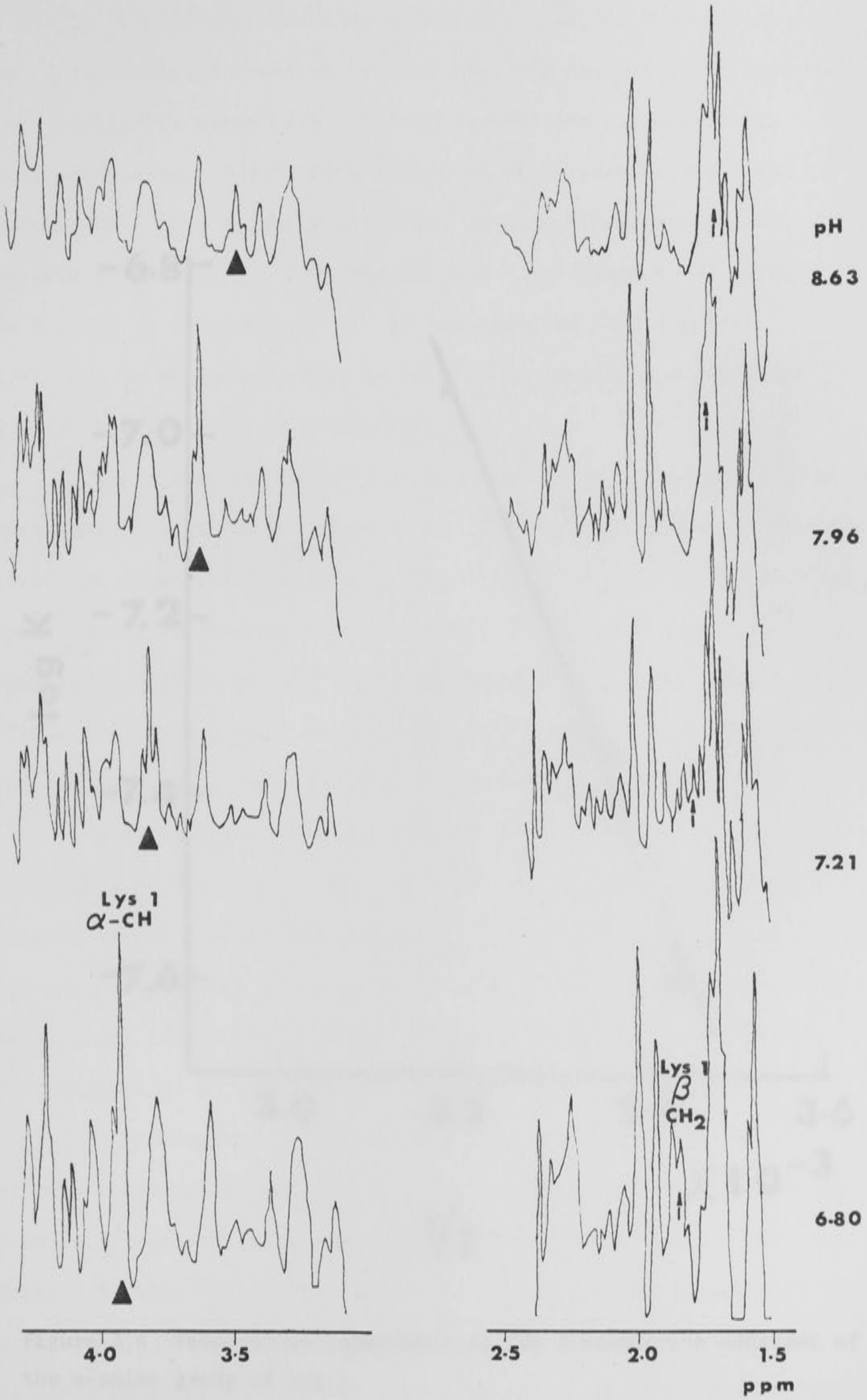


Figure 3.5 Titration of the α-CH and the β-CH_2 resonances of Lys 1. 7.25 mM of RNase A with 0.3 M NaCl at 35°C was used for obtaining the spectra.

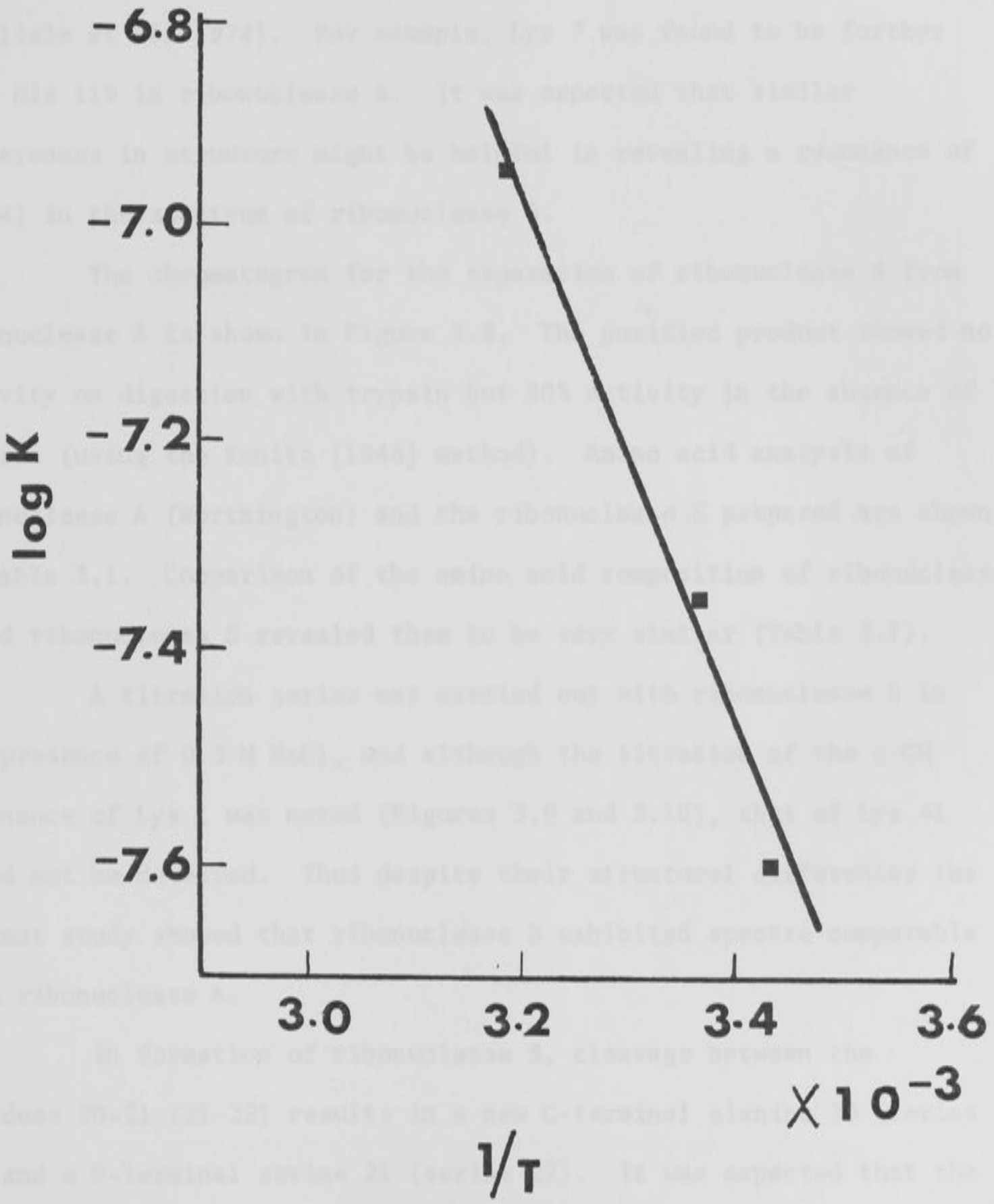


Figure 3.6 Temperature dependance of the dissociation constant of the α-amino group of Lys 1.

and Witkop [1967] indicated that either the 20-21 bond or the 21-22 bond of ribonuclease A was broken and that the product ratio depended on the particular proteinase preparation used for the digestion. Furthermore, x-ray studies have indicated that ribonuclease S and ribonuclease A have slightly different conformational structures [Carlisle *et al.* 1974]. For example, Lys 7 was found to be further from His 119 in ribonuclease S. It was expected that similar differences in structure might be helpful in revealing a resonance of Lys 41 in the spectrum of ribonuclease S.

The chromatogram for the separation of ribonuclease S from ribonuclease A is shown in Figure 3.8. The purified product showed no activity on digestion with trypsin but 80% activity in the absence of trypsin (using the Kunitz [1946] method). Amino acid analysis of ribonuclease A (Worthington) and the ribonuclease S prepared are shown in Table 3.1. Comparison of the amino acid composition of ribonuclease A and ribonuclease S revealed them to be very similar (Table 3.1).

A titration series was carried out with ribonuclease S in the presence of 0.3 M NaCl, and although the titration of the α-CH resonance of Lys 1 was noted (Figures 3.9 and 3.10), that of Lys 41 could not be detected. Thus despite their structural differences the present study showed that ribonuclease S exhibited spectra comparable with ribonuclease A.

In formation of ribonuclease S, cleavage between the residues 20-21 (21-22) results in a new C-terminal alanine 20 (serine 21) and a N-terminal serine 21 (serine 22). It was expected that the titration of this α-CH resonance of serine 21 would be observed in the pH range studied. A pH titration series of Ser-Gly-Gly showed that the α-amino group titrated with a pK of 7.1 at 31°C (Figure 3.11). However, the titration of this function could not be detected in the

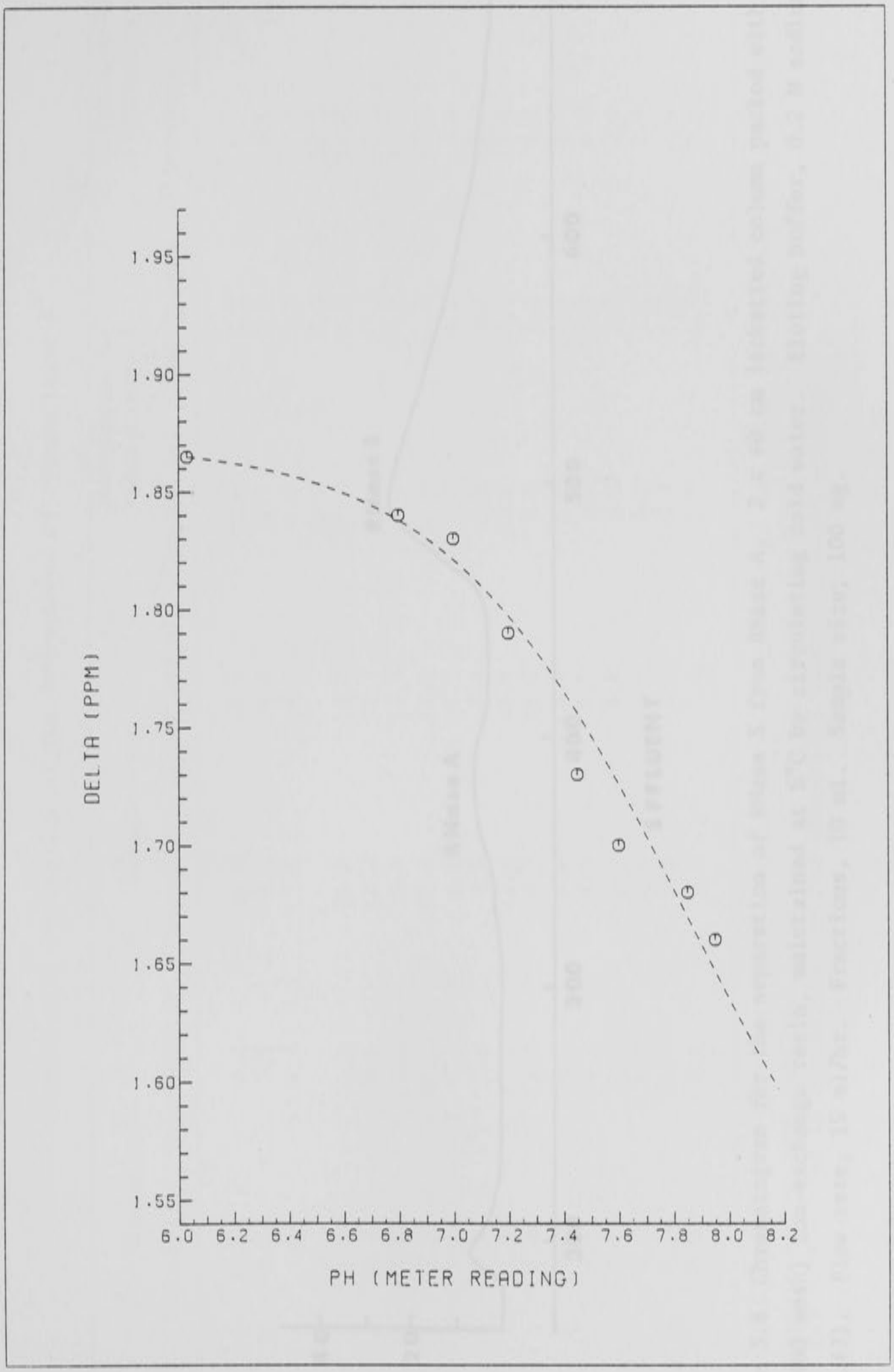


Figure 3.7 Titration curve for the β-CH_2 resonance of Lys 1. The details of the experimental conditions are given in Figure 3.5.

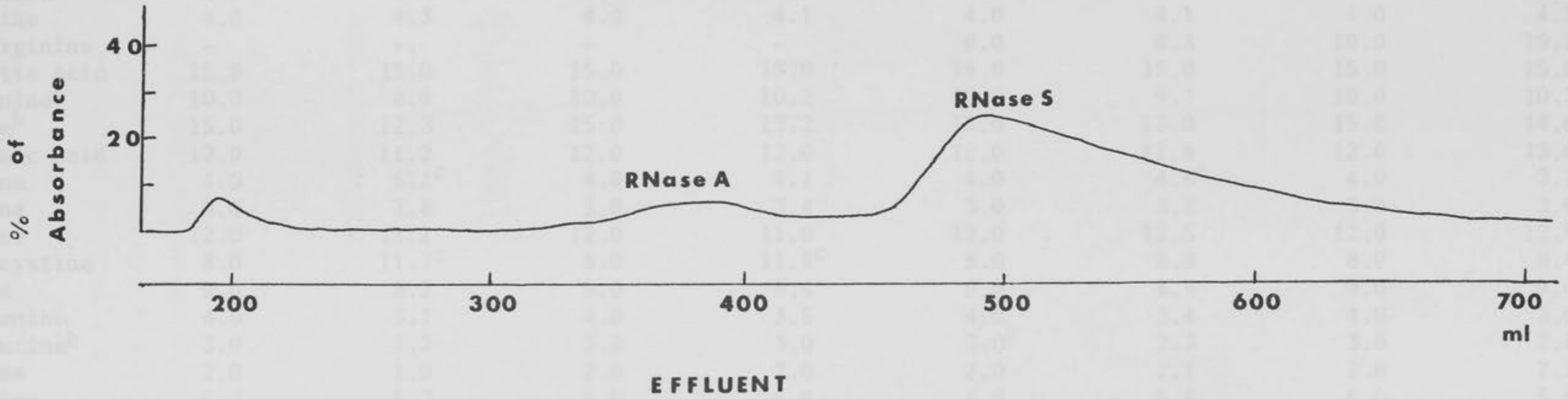


Figure 3.8 Chromatogram for the separation of RNase S from RNase A. 2 x 40 cm jacketted column packed with Bio-Rex 70 (200-400 mesh) ion-exchange resin, maintained at 5°C by circulating cold water. Eluting buffer, 0.2 M sodium phosphate (pH 6.47). Flow rate, 15 ml/hr. Fractions, 10 ml. Sample size, 100 mg.

TABLE 3.1

Amino acid composition of the derivatives of ribonuclease A[a]

	Ribonuclease A		Ribonuclease S		Nonaguanidino ribonuclease A		Decaguanidino ribonuclease A	
	Theoretical	Experimental	Theoretical	Experimental	Theoretical	Experimental	Theoretical	Experimental
Lysine	10.0	9.1	10.0	10.6	1.0	1.1	0	0.4
Histidine	4.0	3.5	4.0	4.0	4.0	3.6	4.0	4.0
Arginine	4.0	4.3	4.0	4.1	4.0	4.1	4.0	4.3
Homoarginine	-	-	-	-	9.0	8.3	10.0	10.1
Aspartic Acid	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.8
Threonine[b]	10.0	8.4	10.0	10.2	10.0	9.7	10.0	10.3
Serine[b]	15.0	12.3	15.0	13.2	15.0	13.0	15.0	14.6
Glutamic Acid	12.0	11.2	12.0	12.0	12.0	11.8	12.0	13.0
Proline	4.0	5.2[c]	4.0	4.1	4.0	4.8[c]	4.0	3.7
Glycine	3.0	2.8	3.0	3.4	3.0	3.2	3.0	3.6[c]
Alanine	12.0	11.2	12.0	11.0	12.0	12.5	12.0	12.8
Half-cystine	8.0	11.7[c]	8.0	11.8[c]	8.0	8.8	8.0	8.4
Valine	9.0	8.2	9.0	8.4	9.0	8.5	9.0	9.0
Methionine	4.0	3.7	4.0	3.5	4.0	3.4	4.0	3.6
Isoleucine[b]	3.0	2.2	3.0	3.0	3.0	2.3	3.0	2.8
Leucine	2.0	1.9	2.0	2.0	2.0	2.1	2.0	2.2
Tyrosine	6.0	5.7	6.0	5.9	6.0	5.8	6.0	5.5
Phenylalanine	3.0	2.9	3.0	2.8	3.0	3.3	3.0	3.2

a The mean of two analyses for each substance is given here. Aspartic acid was taken as 15.0 residues in determining the amount of protein hydrolyzate analysed.

b Correction for destruction in hydrolysis, or for incomplete liberation, not applied.

c Could not be accounted for.

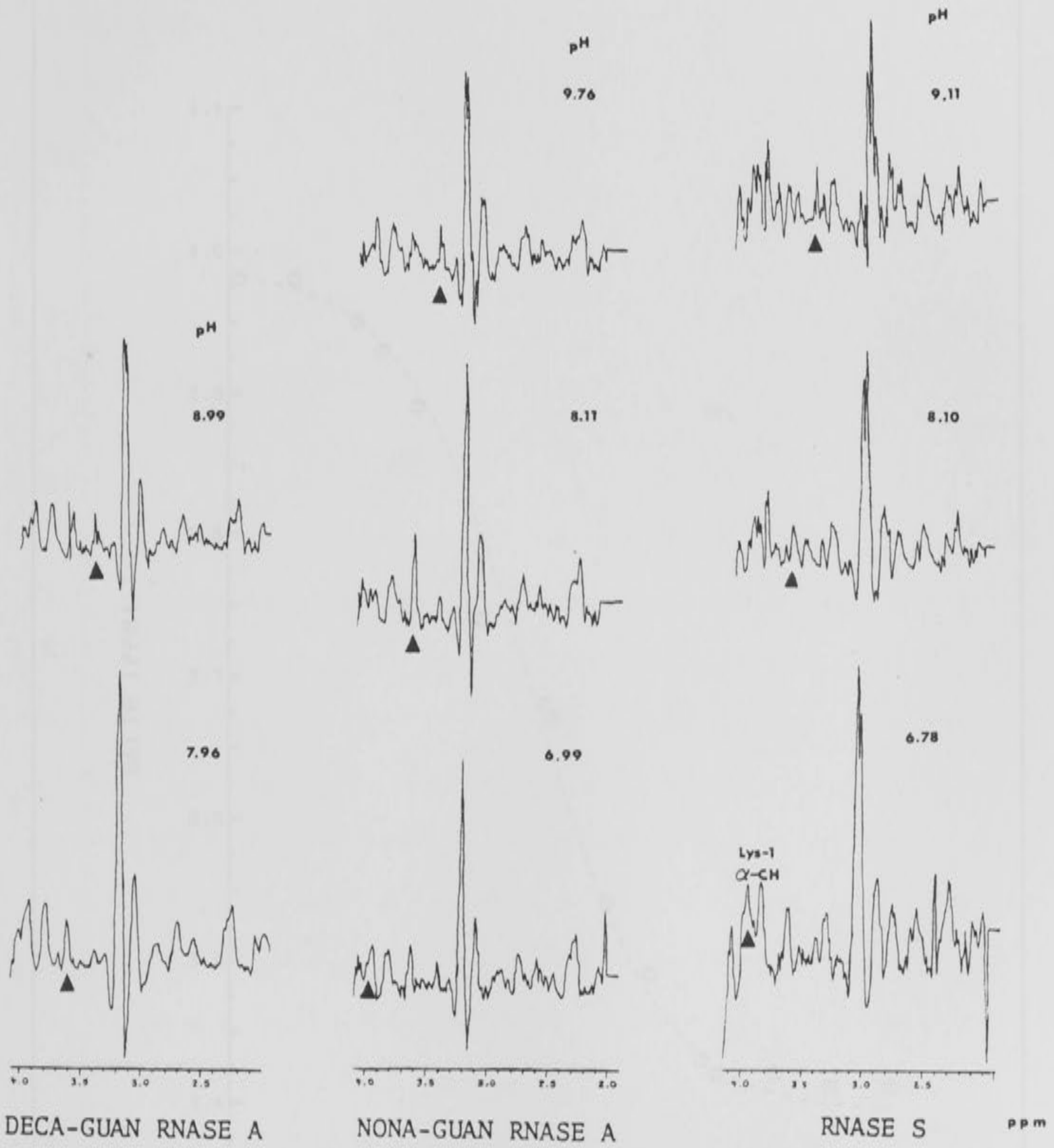


Figure 3.9 Titration of the α-CH resonance of Lys 1 in ribonuclease S and the nona and decaguanidino ribonuclease A. 2.17 mM of the protein solution containing 0.3 M NaCl at 35°C was used for obtaining the spectra.

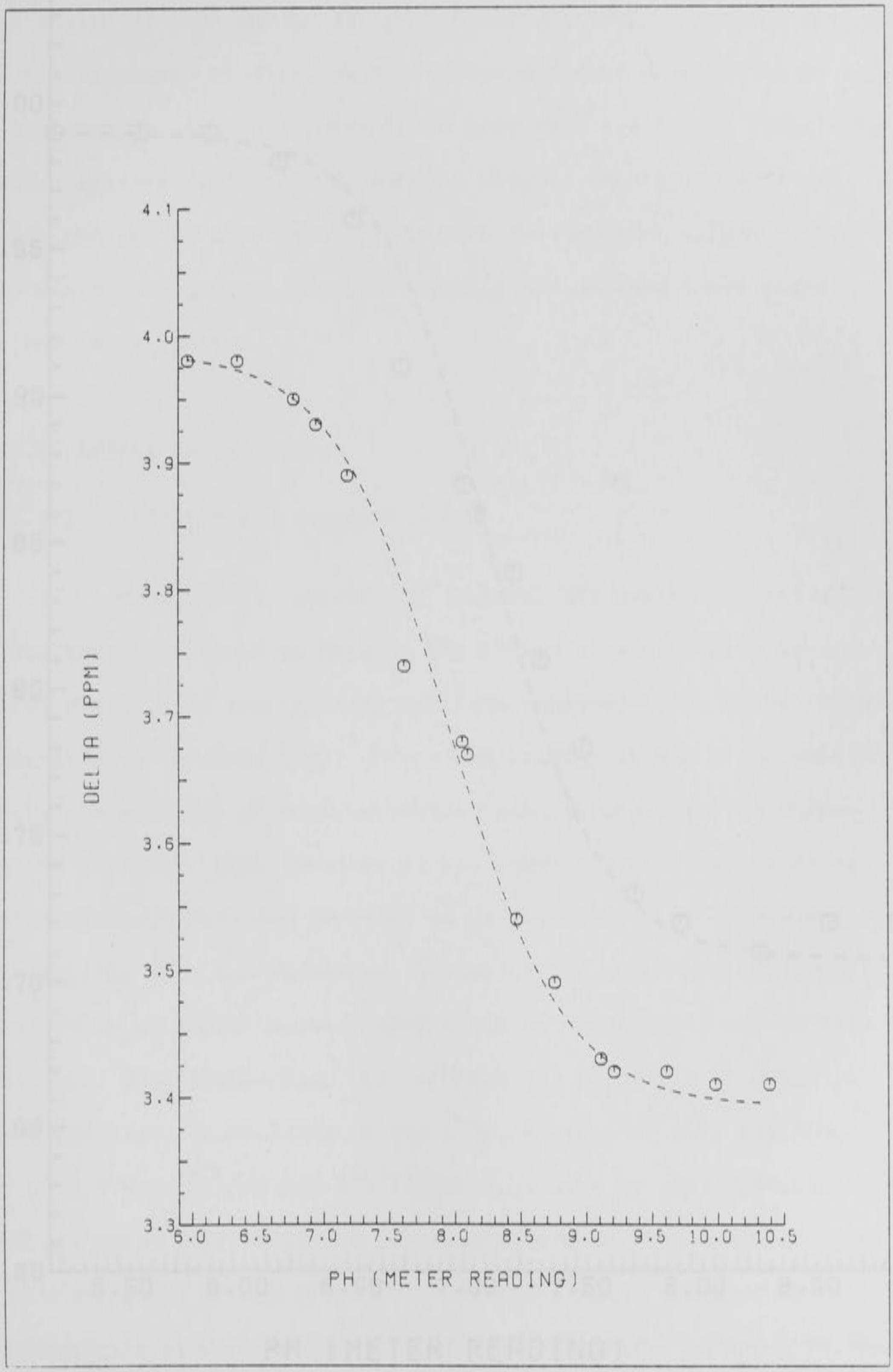


Figure 3.10 A typical titration curve for the α-CH resonance of Lys 1 in ribonuclease S and the nona and decaguanidino ribonuclease A.

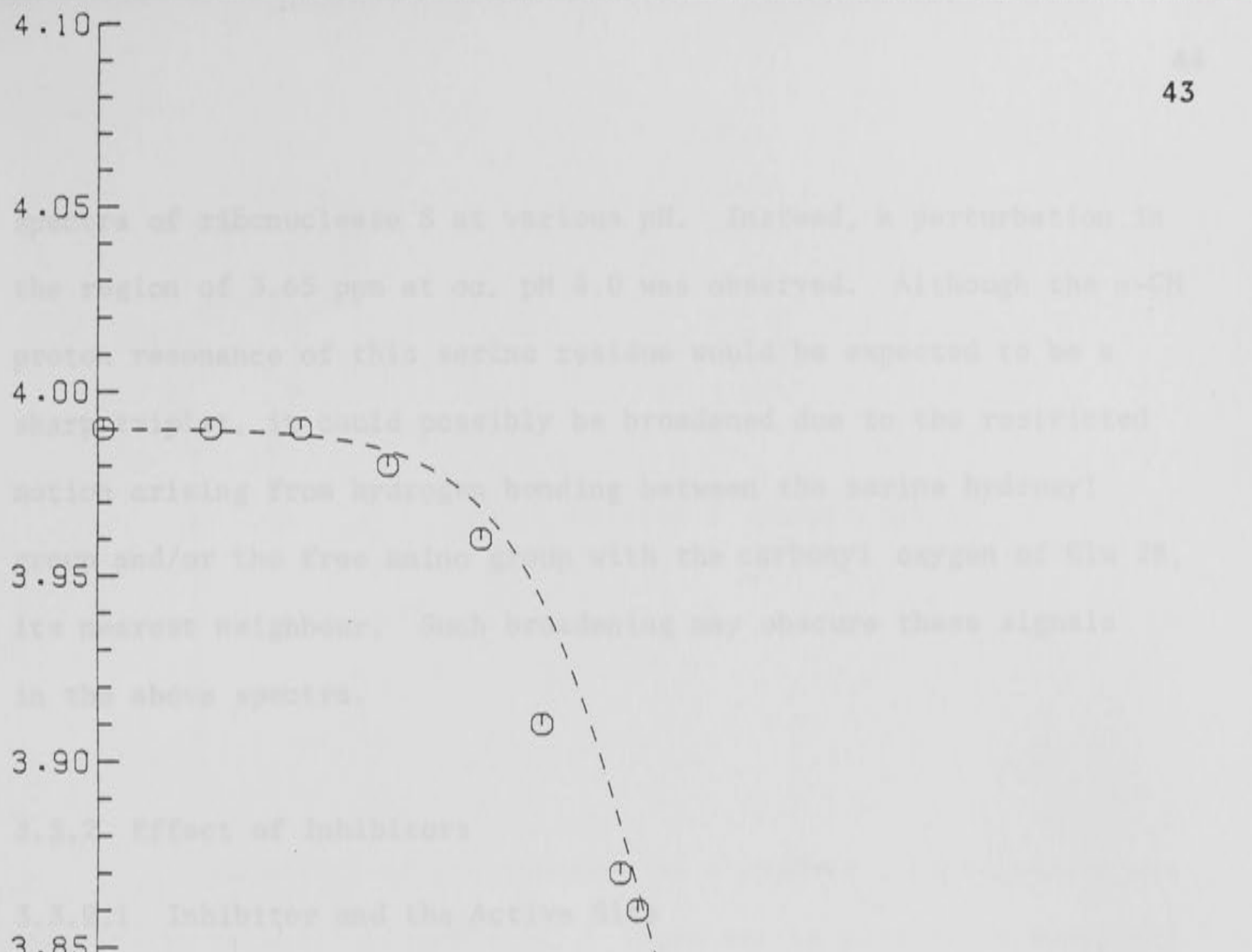


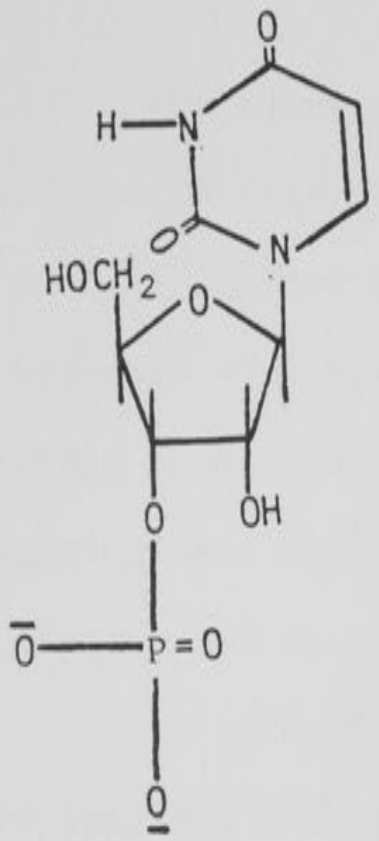
Figure 3.11 Titration curve for the α-CH resonance of Ser in Ser-Gly-Gly. Spectra obtained at 80 MHz using 1% solution of the peptide containing 0.15 M NaCl at a temperature of 31°C.

spectra of ribonuclease S at various pH. Instead, a perturbation in the region of 3.65 ppm at *ca.* pH 8.0 was observed. Although the α-CH proton resonance of this serine residue would be expected to be a sharp triplet, it could possibly be broadened due to the restricted motion arising from hydrogen bonding between the serine hydroxyl group and/or the free amino group with the carbonyl oxygen of Glu 28, its nearest neighbour. Such broadening may obscure these signals in the above spectra.

3.3.2 Effect of Inhibitors

3.3.2.1 Inhibitor and the Active Site

Most anions, polyanions (natural and synthetic) and nucleotides have been found to bind to the active site of ribonuclease A quite readily and this binding has been reported to be competitive [Richards and Wyckoff 1971]. From NMR studies it was shown that the phosphate group in 3'-mononucleotides bind strongly to the enzyme in the dianionic form [Meadows *et al.* 1969]. Since the Lys 41 of ribonuclease A has been reported to be involved in an electrostatic interaction with the phosphoryl oxygen of the substrates and inhibitors bind in an analogous manner, they could be expected to affect this residue. Such interaction may increase the pK of Lys 41 which in turn may cause deshielding of the ε-CH_2 protons of this residue. At pH 6.6 both His 12 and His 119 will be more or less deprotonated and the major contributor to interaction between the inhibitor and the enzyme will be the positively charged ε-amino group of Lys 41, although the binding of the inhibitor to the enzyme is known to fall dramatically above and below pH 5.5 [Richards and Wyckoff 1971].



Uridine 3'-monophosphate 3.2

The effect of the addition of 3'-UMP to ribonuclease A was studied at pH 6.6 (Figure 3.12). There was no noticeable downfield shift of any resonance around 3.0 ppm when this addition was carried out (Figure 3.12), but instead, the ε-CH_2 resonance envelope showed a progressive splitting. Along with Lys 41, -7 and -66 are other lysine residues that are reported to be present in or near the active site (section 3.1) and these residues may also be affected by 3'-UMP binding to the active site of this enzyme. It is not known for certain whether this splitting is due to the interaction of one or more of these residues with the inhibitor or not. These interactions are also capable of broadening resonances due to an intermediate rate of exchange between the bound and unbound forms on the NMR time scale (the C_2-H resonance of His 12 is broadened on inhibitor binding to ribonuclease A [Ruterjans and Witzel 1969]). So the failure to observe any downfield shift does not definitely establish the absence of any such interaction between Lys 41 and the inhibitor. Spin decoupling experiments were also carried out in the presence of 3'-UMP by irradiating the δ-CH_2 protons of the lysine residues, but without success.

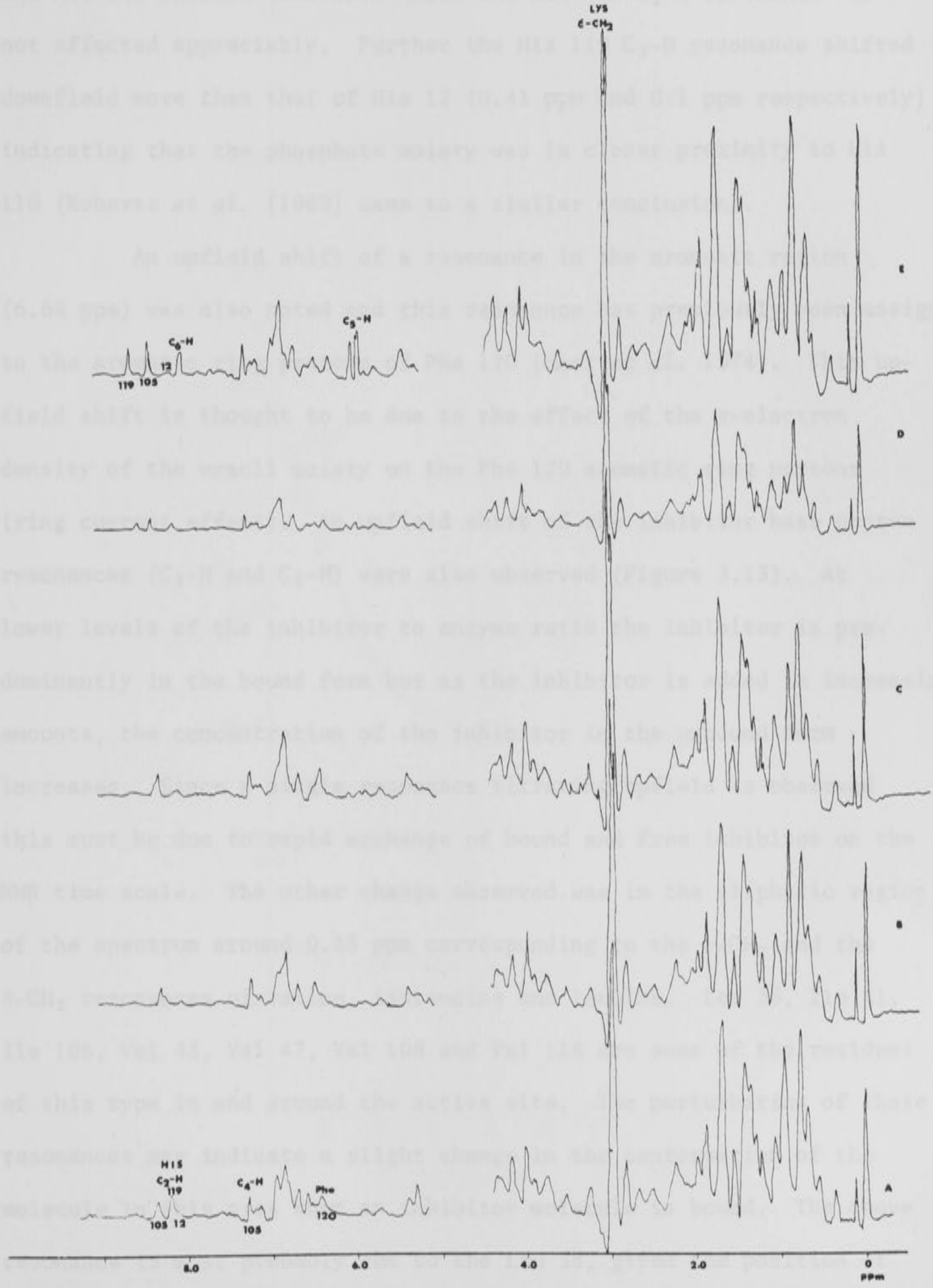


Figure 3.12 Addition of 3'-UMP to ribonuclease A studied at 270 MHz. The inhibitor addition to 7.25 mM of ribonuclease A containing 0.3 M NaCl was carried out at 22°C. The inhibitor added was from a 1 M stock solution in ^2H_2O.
(A) 0 mM; (B) 1 mM; (C) 2 mM; (D) 4 mM; (E) 8 mM.

The other effects observed on addition of inhibitor are summarized in Figures 3.12 and 3.13. The C_2-H resonances of His 12 and His 119 shifted downfield while the His 105 C_2-H resonance was not affected appreciably. Further the His 119 C_2-H resonance shifted downfield more than that of His 12 (0.41 ppm and 0.1 ppm respectively) indicating that the phosphate moiety was in closer proximity to His 119 (Roberts *et al.* [1969] came to a similar conclusion).

An upfield shift of a resonance in the aromatic region (6.68 ppm) was also noted and this resonance has previously been assigned to the aromatic ring protons of Phe 120 [Haar *et al.* 1974]. This upfield shift is thought to be due to the effect of the π-electron density of the uracil moiety on the Phe 120 aromatic ring protons (ring current effect). An upfield shift of the inhibitor base proton resonances (C_5-H and C_6-H) were also observed (Figure 3.13). At lower levels of the inhibitor to enzyme ratio the inhibitor is predominantly in the bound form but as the inhibitor is added in increasing amounts, the concentration of the inhibitor in the unbound form increases. Since a single resonance titrating upfield is observed this must be due to rapid exchange of bound and free inhibitor on the NMR time scale. The other change observed was in the aliphatic region of the spectrum around 0.85 ppm corresponding to the γ-CH_3 and the δ-CH_3 resonances of valine, isoleucine and leucine. Leu 35, Ile 81, Ile 106, Val 43, Val 47, Val 108 and Val 118 are some of the residues of this type in and around the active site. The perturbation of these resonances may indicate a slight change in the conformation of the molecule in this area when an inhibitor molecule is bound. The above resonance is most probably due to the Leu 35, given the position of this residue.

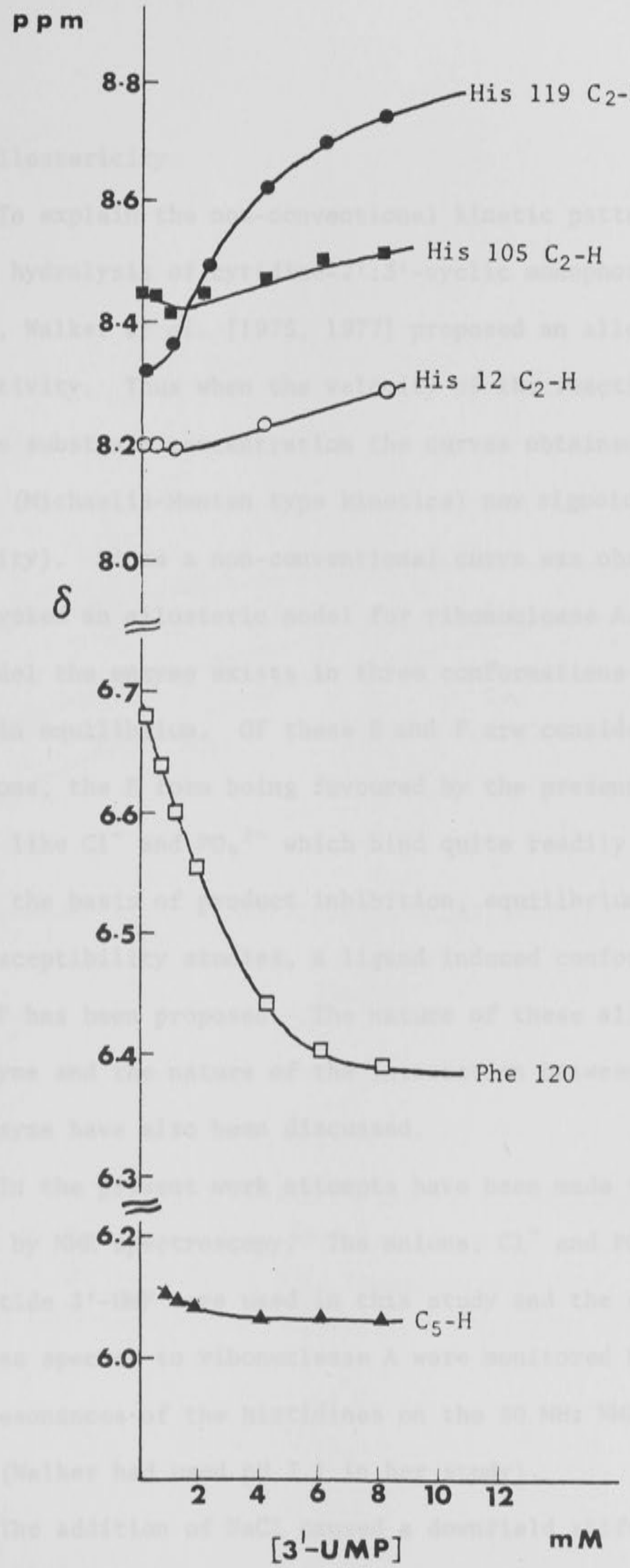


Figure 3.13 Chemical shifts of the C_2-H resonances of histidines, aromatic proton resonances of Phe 120 and C_5-H resonance of the uracil base of 3'-UMP, as a function of added inhibitor concentration. The experimental details are described in Figure 3.12.

3.3.2.2 Allostericity

To explain the non-conventional kinetic patterns observed during the hydrolysis of cytidine-2':3'-cyclic monophosphate by ribonuclease A, Walker *et al.* [1975, 1977] proposed an allosteric model for its activity. Thus when the velocity of the reaction was plotted against the substrate concentration the curves obtained were neither hyperbolic (Michaelis-Menten type kinetics) nor sigmoidal (positive cooperativity). Since a non-conventional curve was observed these workers invoked an allosteric model for ribonuclease A. According to this model the enzyme exists in three conformations E, F and G which are in equilibrium. Of these E and F are considered to be active conformations, the F form being favoured by the presence of nucleotides and anions like Cl^- and PO_4^{3-} which bind quite readily to the allosteric sites. On the basis of product inhibition, equilibrium dialysis and trypsin susceptibility studies, a ligand induced conformational change from E to F has been proposed. The nature of these allosteric sites in the enzyme and the nature of the interaction between the nucleotides and the enzyme have also been discussed.

In the present work attempts have been made to observe this phenomenon by NMR spectroscopy. The anions, Cl^- and PO_4^{3-} and the mononucleotide 3'-UMP were used in this study and the effect of adding these species to ribonuclease A were monitored by observing the C_2-H resonances of the histidines on the 80 MHz NMR instrument at pH 6.6 (Walker had used pH 7.1 in her study).

The addition of NaCl caused a downfield shift of the C_2-H resonances of His 12, -105 and -119 (Figure 3.14). From the extent of the downfield shifts it is evident that the active site histidines (His 12 - 0.38 ppm and His 119 - 0.25 ppm) are affected more than the one on the surface of the enzyme (His 105 - 0.14 ppm). The presence

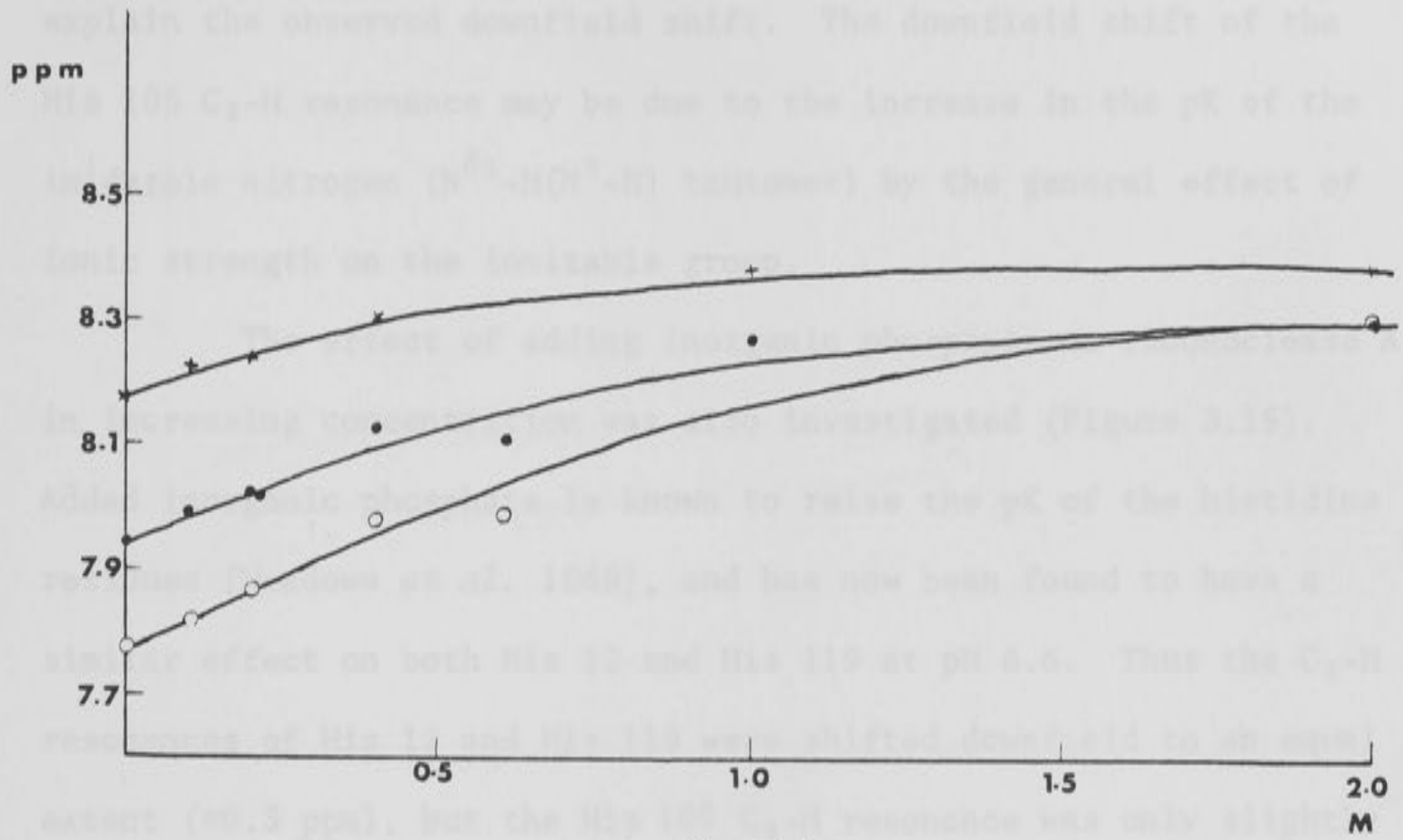


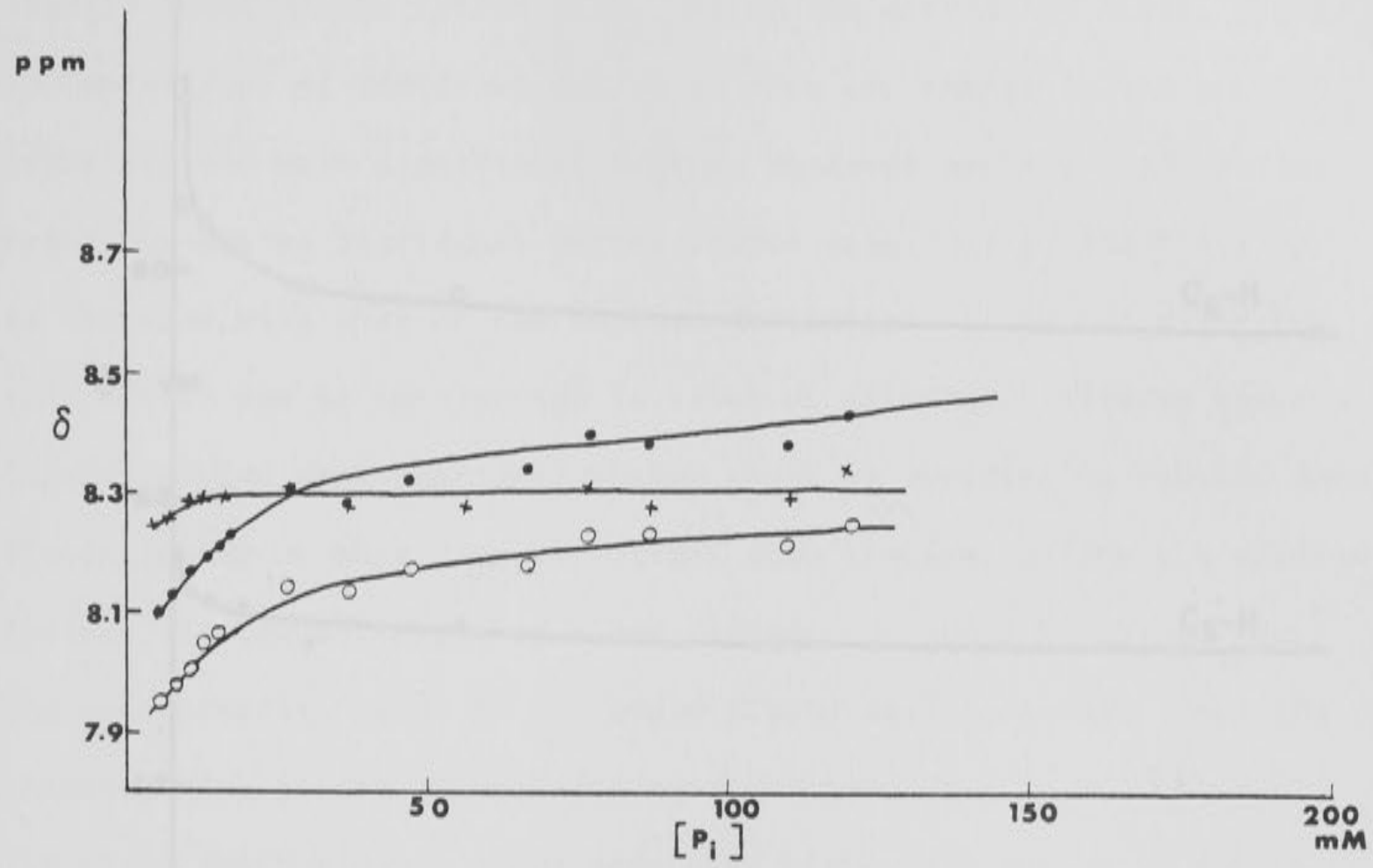
Figure 3.14 The chemical shifts of the C_2-H resonances of histidines studied at 80 MHz as function of total added NaCl to RNase A. RNase A = 8.7 mM; pH = 6.6 ± 0.1; Temperature = 37°C. 5 M stock solution of NaCl in ^2H_2O. X - His 105; ● - His 119; O - His 12.

of one or more negatively charged chloride ions in the active site of ribonuclease A change its positive character through electrostatic interactions [Rüterjans and Witzel 1969] which is responsible for the increase in pK of these active site histidine residues and hence explain the observed downfield shift. The downfield shift of the His 105 C_2-H resonance may be due to the increase in the pK of the imidazole nitrogen ($N^{\delta 1}$-H(N^{π}-H) tautomer) by the general effect of ionic strength on the ionizable group.

The effect of adding inorganic phosphate to ribonuclease A in increasing concentration was also investigated (Figure 3.15). Added inorganic phosphate is known to raise the pK of the histidine residues [Meadows *et al.* 1969], and has now been found to have a similar effect on both His 12 and His 119 at pH 6.6. Thus the C_2-H resonances of His 12 and His 119 were shifted downfield to an equal extent (≃0.3 ppm), but the His 105 C_2-H resonance was only slightly affected (≃0.05 ppm). Since this addition was carried out at constant ionic strength, the larger shifts of the active site histidine resonances as compared with the surface histidine resonances indicates that there is an electrostatic interaction between the phosphate anion and the positively charged active site.

The addition of 3'-UMP to ribonuclease A was studied at the same pH as was employed for that in section 3.3.2.1 (i.e. pH = 6.6) but at higher concentration levels of the inhibitor to the enzyme. Analogous results were obtained and these are summarized in Figure 3.16.

The effect of the addition of mononucleotide inhibitor and anions to ribonuclease A as monitored by NMR are shown in Figure 3.14, 3.15 and 3.16. When the chemical shifts of resonances affected by this addition were plotted against the concentrations of the added



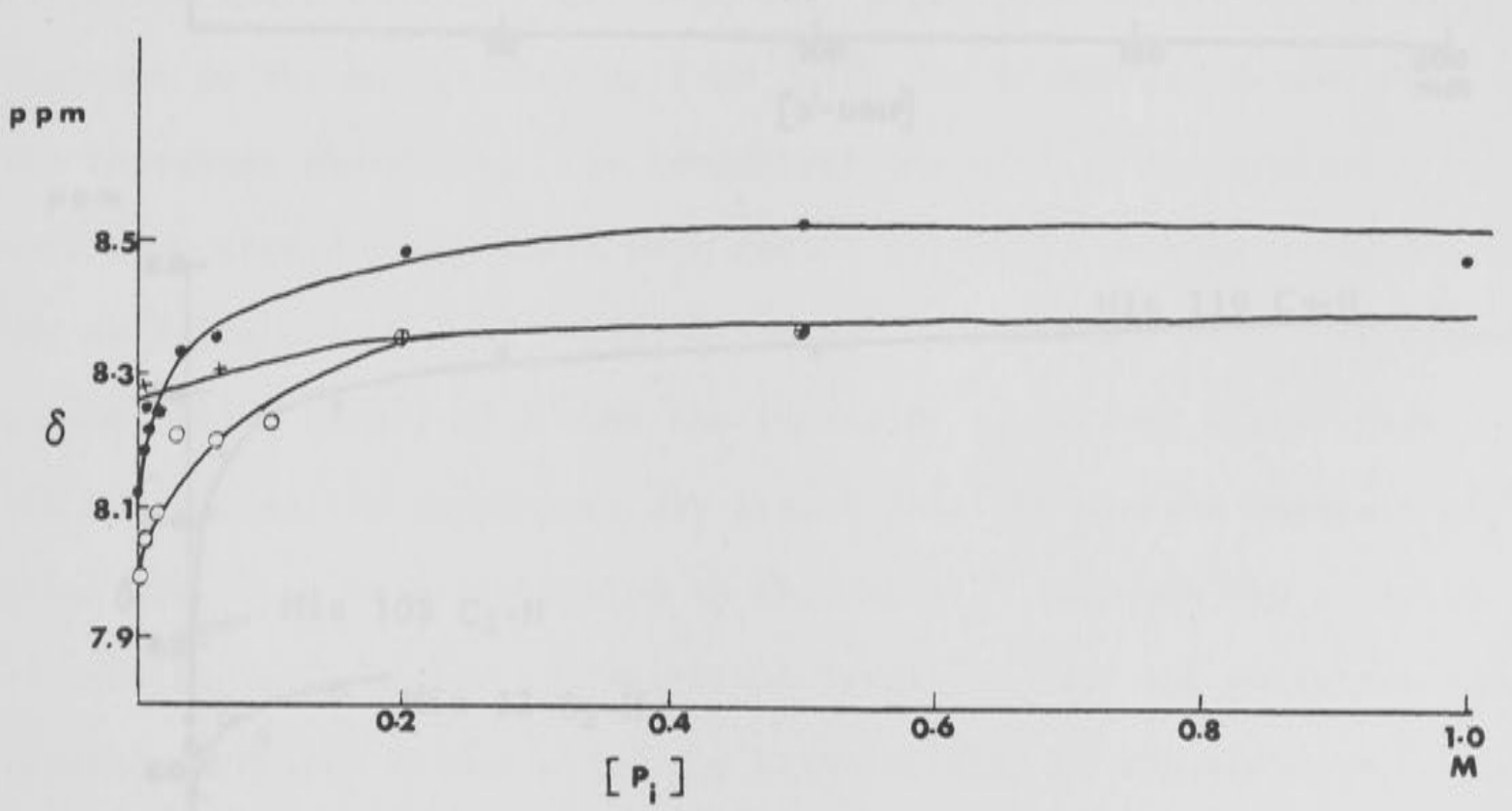


Figure 3.15 The effect of addition of inorganic phosphate (Pi) to ribonuclease A studied at 80 MHz by monitoring the C_2-H resonances of histidine, RNase A (upper trace) = 8.7 mM; RNase A (lower trace) = 7.25 mM; NaCl = 0.3 M; pH = 6.6 ± 0.1; Temp = 37°C; Pi = 1 M stock solution.

X - His 105; ● - His 119; O - His 12.

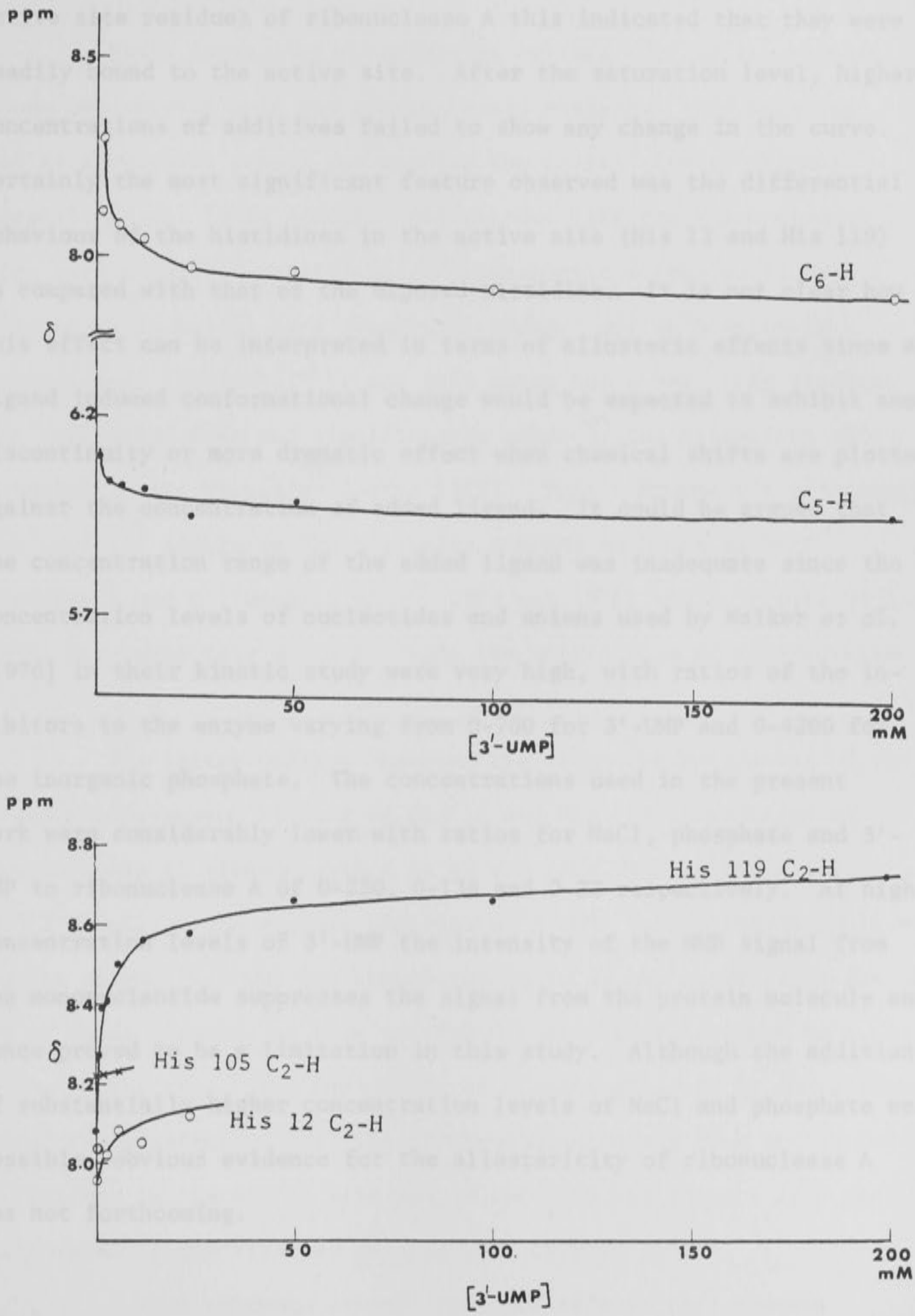


Figure 3.16 The effect of 3'-UMP addition to ribonuclease A studied at 80 MHz. RNase A = 8.7 mM; NaCl = 0.3 M; pH = 6.6 ± 0.1; Temp = 37°C. 3'-UMP = 1 M stock solution.

nucleotides and anions, hyberbolic curves were obtained. Since the initial addition of nucleotide and anions had a greater effect on the active site residues of ribonuclease A this indicated that they were readily bound to the active site. After the saturation level, higher concentrations of additives failed to show any change in the curve. Certainly the most significant feature observed was the differential behaviour of the histidines in the active site (His 12 and His 119) as compared with that of the exposed histidine. It is not clear how this effect can be interpreted in terms of allosteric effects since a ligand induced conformational change would be expected to exhibit some discontinuity or more dramatic effect when chemical shifts are plotted against the concentration of added ligand. It could be argued that the concentration range of the added ligand was inadequate since the concentration levels of nucleotides and anions used by Walker *et al.* [1976] in their kinetic study were very high, with ratios of the inhibitors to the enzyme varying from 0-700 for 3'-UMP and 0-4200 for the inorganic phosphate. The concentrations used in the present work were considerably lower with ratios for NaCl, phosphate and 3'-UMP to ribonuclease A of 0-230, 0-138 and 0-23 respectively. At higher concentration levels of 3'-UMP the intensity of the NMR signal from the mononucleotide suppresses the signal from the protein molecule and hence proved to be a limitation in this study. Although the addition of substantially higher concentration levels of NaCl and phosphate were possible, obvious evidence for the allostericity of ribonuclease A was not forthcoming.

3.3.3 Effect of Addition of Paramagnetic Anions to Ribonuclease A

3.3.3.1 Potassium hexacyanochromate (III)

Since paramagnetic lanthanide cations bind to the carboxyl groups of aspartic acid and glutamic acid residues, they have been used as structural probes for mapping the active site of enzymes [Campbell *et al.* 1975a and b]. For enzymes like the ribonucleases which have a positive active site, paramagnetic anions would be expected to be useful as a structural probe. One such anion is chromicyanide ($[Cr(CN)_6]^{3-}$), and this anion has a very long electron spin relaxation time and significantly enhances the relaxation rates of nuclei in its proximity [James 1975]. This compound has previously been used for assigning the histidine protons in human carbonic anhydrase B and C [Campbell *et al.* 1974 and 1975] while Inagaki *et al.* [1979] have used this anion to study the active site of ribonuclease A.

The latter workers observed the selective broadening of the C_2 and the C_4 proton signals of His 12 and His 119 on addition of chromicyanide to ribonuclease A at pH 4.0 (and 30°C in the presence of 0.3 M NaCl). The gradual addition of 3'-UMP to a solution containing the enzyme (1.5 mM) and chromicyanide (0.45 mM) resulted in sharpening of these signals, so they concluded that this anion must be bound to the active site. The broadening of signals observed in the aliphatic end of the spectrum on addition of chromicyanide had been attributed to residues in the active site but no specific assignments were made. Furthermore no quantitative studies were carried out.

A more thorough attempt has been made in this present study to map the active site of ribonuclease A using chromicyanide. It was expected that the broadening of resonances by chromicyanide could

be used for assigning the resonances from residues in the active site, especially those of Lys 41.

Chromicyanide was added to ribonuclease A in the presence of 0.3 M NaCl at pH 4.0 and temperatures of 25°C and 35°C, and the results are shown in Figure 3.17. At both temperatures the C_2-H resonances of His 12 and His 119 were broadened and the rate of broadening of the C_2-H resonance of His 119 was found to be greater than that of the His 12 C_2-H resonance. Neither the His 105 C_2-H and C_4-H nor the His 48 C_2-H resonances were affected but noticeable broadening was observed in one of the peaks of the lysine ε-CH_2 resonance envelope. Since both Lys 7 and Lys 41 are situated at the active site this resonance could possibly originate from either of them. On titration this resonance was found to have a pK typical of normal lysine residues and hence was tentatively assigned to that of Lys 7. Although the nature of the interaction between the chromicyanide anion and the active site are not clearly understood [see Campbell *et al.* 1974] it is apparent from the present study (i.e. the relative rates of broadening) that the bound chromicyanide anion is closer to His 119 than to His 12. Analogous behaviour has been observed previously when 3'-UMP was used as an inhibitor. A possible site for the chromicyanide binding may be the N1 nitrogen of His 119 ($N^{\varepsilon 2}$-H(N^{τ}-H) tautomer). His 119 is the only histidine residue in ribonuclease A in which the N1 nitrogen is the titrating group [Walters and Allerhand 1980]. Thus the N1 nitrogen of His 119 will be positively charged at this pH and can readily bind the negatively charged chromicyanide anion. Some resonances in the aliphatic region were also observed to be broadened in these spectra.

In order to observe the effect of chromicyanide on the C_4-H resonances of the histidines in the active site of ribonuclease A,

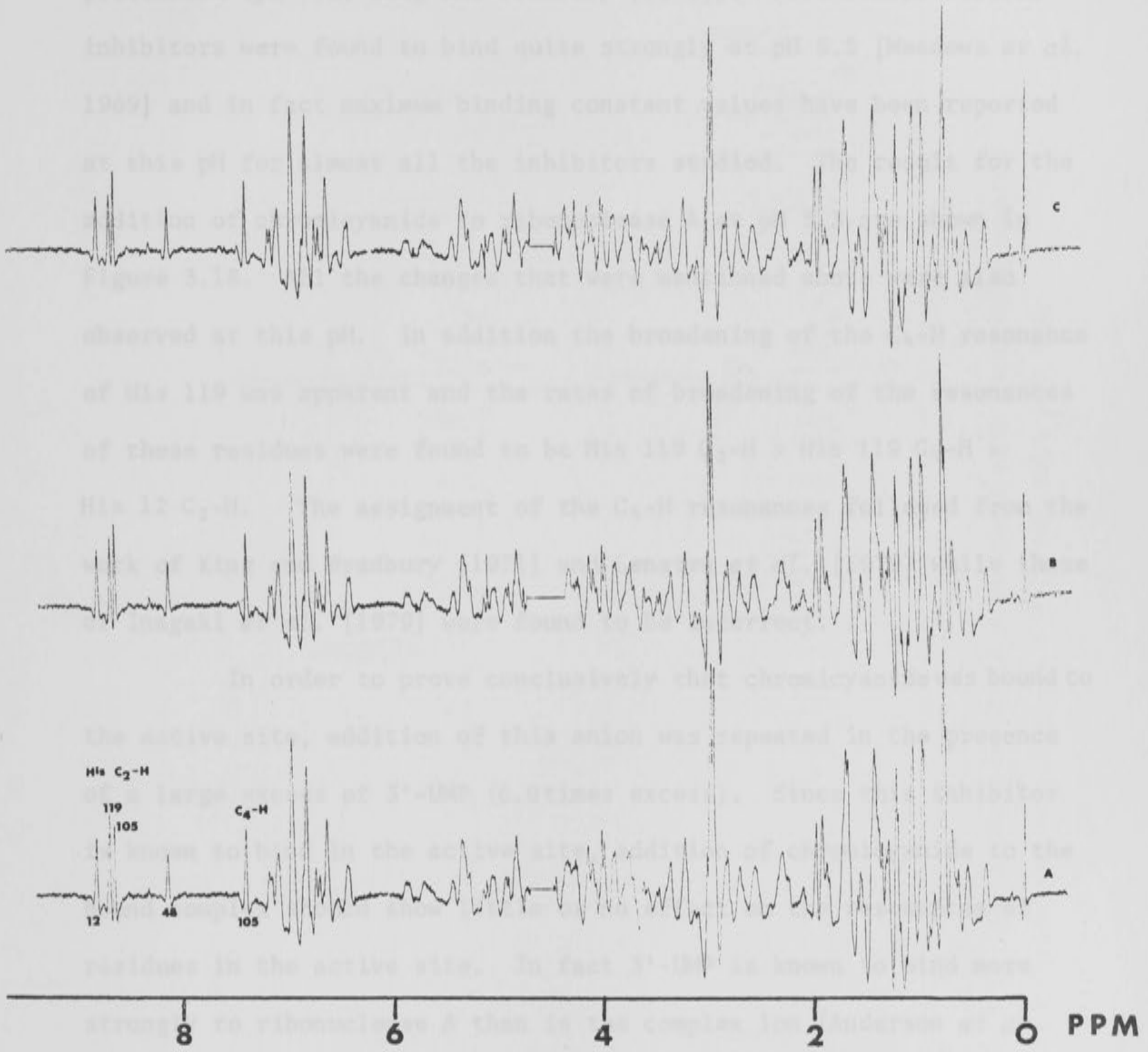


Figure 3.17 The effect of chromicyanide addition to ribonuclease A studied at 270 MHz. 7.25 mM of the protein solution containing 0.3 M NaCl at pH 4.0 and 35°C was used for this experiment. 1 mM stock solution of chromicyanide in ^2H_2O was used for carrying out these additions. A -0μM; B - 40 μM; C - 80 μM.

addition of this anion was studied at pH 5.3 where these resonances were clearly seen. At this pH His 119 will still be protonated (pK 5.8, King and Bradbury [1971]) whereas His 12 will only be partially protonated (pK 5.2, King and Bradbury [1971]). Furthermore various inhibitors were found to bind quite strongly at pH 5.5 [Meadows *et al.* 1969] and in fact maximum binding constant values have been reported at this pH for almost all the inhibitors studied. The result for the addition of chromicyanide to ribonuclease A at pH 5.3 are shown in Figure 3.18. All the changes that were mentioned above were also observed at this pH. In addition the broadening of the C_4-H resonance of His 119 was apparent and the rates of broadening of the resonances of these residues were found to be His 119 C_2-H > His 119 C_4-H > His 12 C_2-H. The assignment of the C_4-H resonances followed from the work of King and Bradbury [1971] and Lenstra *et al.* [1979] while those of Inagaki *et al.* [1979] were found to be incorrect.

In order to prove conclusively that chromicyanide was bound to the active site, addition of this anion was repeated in the presence of a large excess of 3'-UMP (6.9 times excess). Since this inhibitor is known to bind in the active site, addition of chromicyanide to the bound complex should show little or no effect on the resonances of residues in the active site. In fact 3'-UMP is known to bind more strongly to ribonuclease A than is the complex ion [Anderson *et al.* 1968; Inagaki *et al.* 1979]. The results for the addition of chromicyanide to ribonuclease A in the presence of excess 3'-UMP are illustrated in Figure 3.19. Not surprisingly the rates of broadening of the critical resonances were lowered in this case (Figure 3.20) and no evidence was obtained for the binding of chromicyanide to the exposed and positively charged lysine ε-amino groups or arginine δ-guanidino groups. However at higher concentrations of

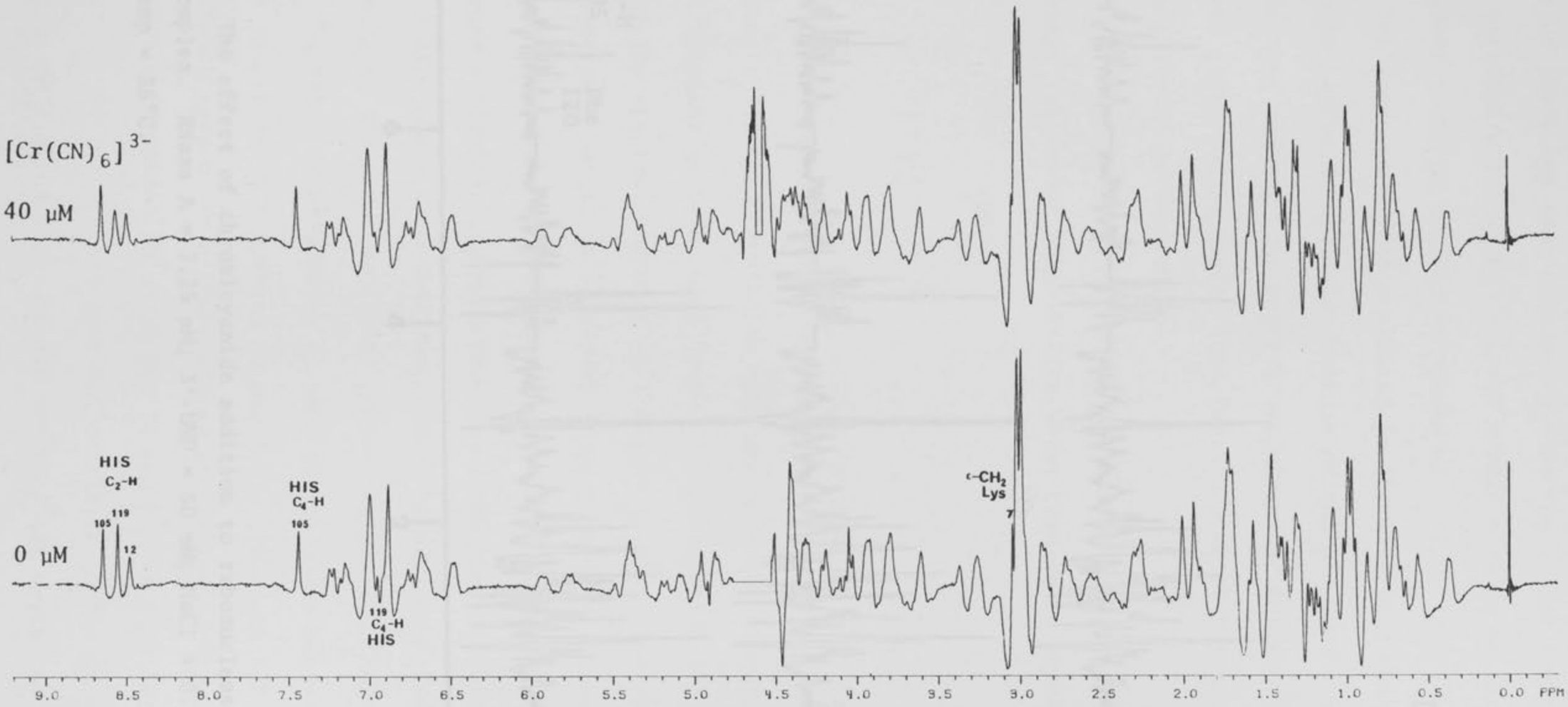


Figure 3.18 The spectra of RNase A obtained with increasing amounts of added chromicyanide. RNase A = 7.25 mM; NaCl = 0.3 M; Temperature = 35°C; pH = 5.3.

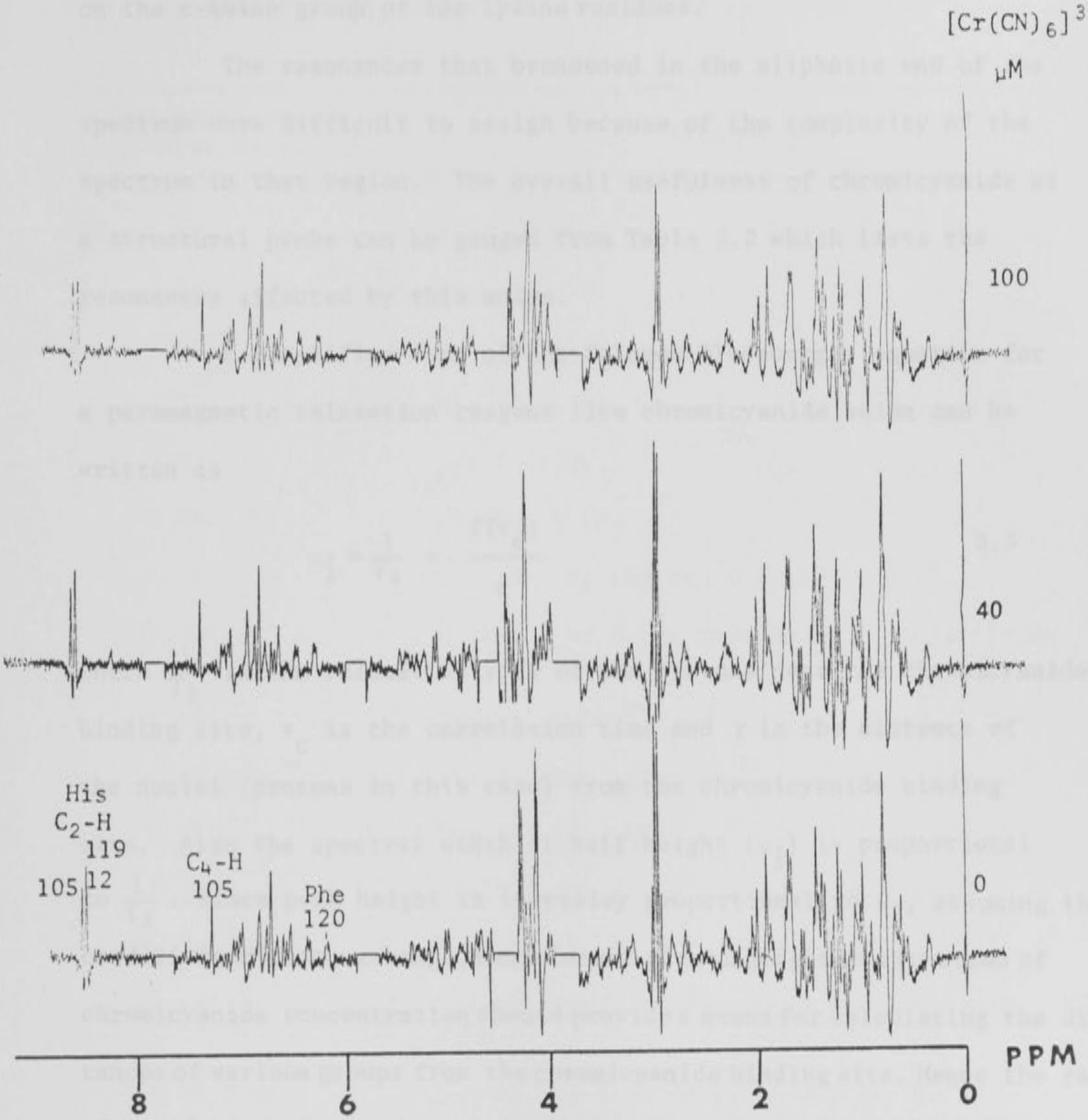


Figure 3.19 The effect of chromicyanide addition to ribonuclease A-inhibitor complex. RNase A = 7.25 mM; 3'-UMP = 50 mM; NaCl = 0.3 M; pH = 5.3; Temp = 35°C.

chromicyanide there was some broadening observed in the lysine ε-CH_2 resonance envelope which might indicate a weak binding of chromicyanide on the ε-amino group of the lysine residues.

The resonances that broadened in the aliphatic end of the spectrum were difficult to assign because of the complexity of the spectrum in that region. The overall usefulness of chromicyanide as a structural probe can be gauged from Table 3.2 which lists the resonances affected by this anion.

A simplified form of the Solomon-Bloembergen equation for a paramagnetic relaxation reagent like chromicyanide anion can be written as

$$\nu_{\frac{1}{2}} \propto \frac{1}{T_2} = \frac{f(\tau_c)}{r^6} \qquad 3.3$$

where $\frac{1}{T_2}$ is the relaxation rate of the protons near the chromicyanide binding site, τ_c is the correlation time and r is the distance of the nuclei (protons in this case) from the chromicyanide binding site. Also the spectral width at half height ($\nu_{\frac{1}{2}}$) is proportional to $\frac{1}{T_2}$. Since peak height is inversley proportional to $\nu_{\frac{1}{2}}$, assuming the conditions of fast exchange, measurement of peak height as a function of chromicyanide concentration should provide a means for calculating the distances of various groups from the chromicyanide binding site. Hence the rates of broadening of the above mentioned proton signals were determined.

The rates of broadening of some of the above mentioned resonances are shown in Figures 3.20 and 3.21. The peak heights were normalised with respect to a sharp signal at 6.92 ppm corresponding to those of the tyrosines (C^{ε}-H of Tyr 76 and C^{δ}-H + C^{ε}-H of Tyr 92 [Lenstra *et al.* 1979]). This peak did not shift in the pH range studied (i.e. pH 4.0 and 5.3) and did not show any change in its

TABLE 3.2

Effect of chromicyanide addition to ribonuclease A[a]

Chemical shift of resonances affected	Assignments
8.58	His 119 C_2-H
8.50	His 12 C_2-H
6.96	His 119 C_4-H
6.70	His 12 C_4-H
3.04	Lys 7 ε-CH_2
3.03-2.98	Lysine ε-CH_2
1.76-1.70	Lys 7 δ-CH_2 and other lysine δ-CH_2[b]
1.70-1.60	Lysine β or δ-CH_2 resonances[b]; Leu 35 β-CH_2 and γ-CH[b]
1.55	Lysine γ-CH_2[b]
1.38-1.26	Ala 4, 109, 122 β-CH_3[b]
1.26	Thr 36, 45 γ-CH_3[b]
1.14	Ile 81, 106 γ-CH_2[b]; Thr 45, 36 γ-CH_3[b]
1.00-0.95	Val 43, 47, 54, 57, 108, 118 γ-CH_3[b]
0.93	Ile 81, 106 γ-CH_3[b]; Leu 35 δ-CH_3[b]; Ile 81, 106 δ-CH_3[b]
0.75-0.64	Resonances from residues mostly CH_3 from Leu, Ile, Val arising from either due to ring current effects and or from the three-dimensional structure of the protein molecule[b]

a Ribonuclease A = 7.25 mM; pH = 5.3; NaCl = 0.3 M; Temp = 35°C. The concentrations of chromicyanide to cause a measurable effect in the above mentioned resonances varied from as low as 5 µM to as high as 100 µM; thereafter at higher concentrations of chromicyanide a general broadening of all the resonances was observed.

b These assignments are tentative based on the assignments for amino acid resonances from Bundi and Wüthrich [1979]. The residue number implies that they are in and around the active site as inferred from the three-dimensional structure of ribonuclease S (model).

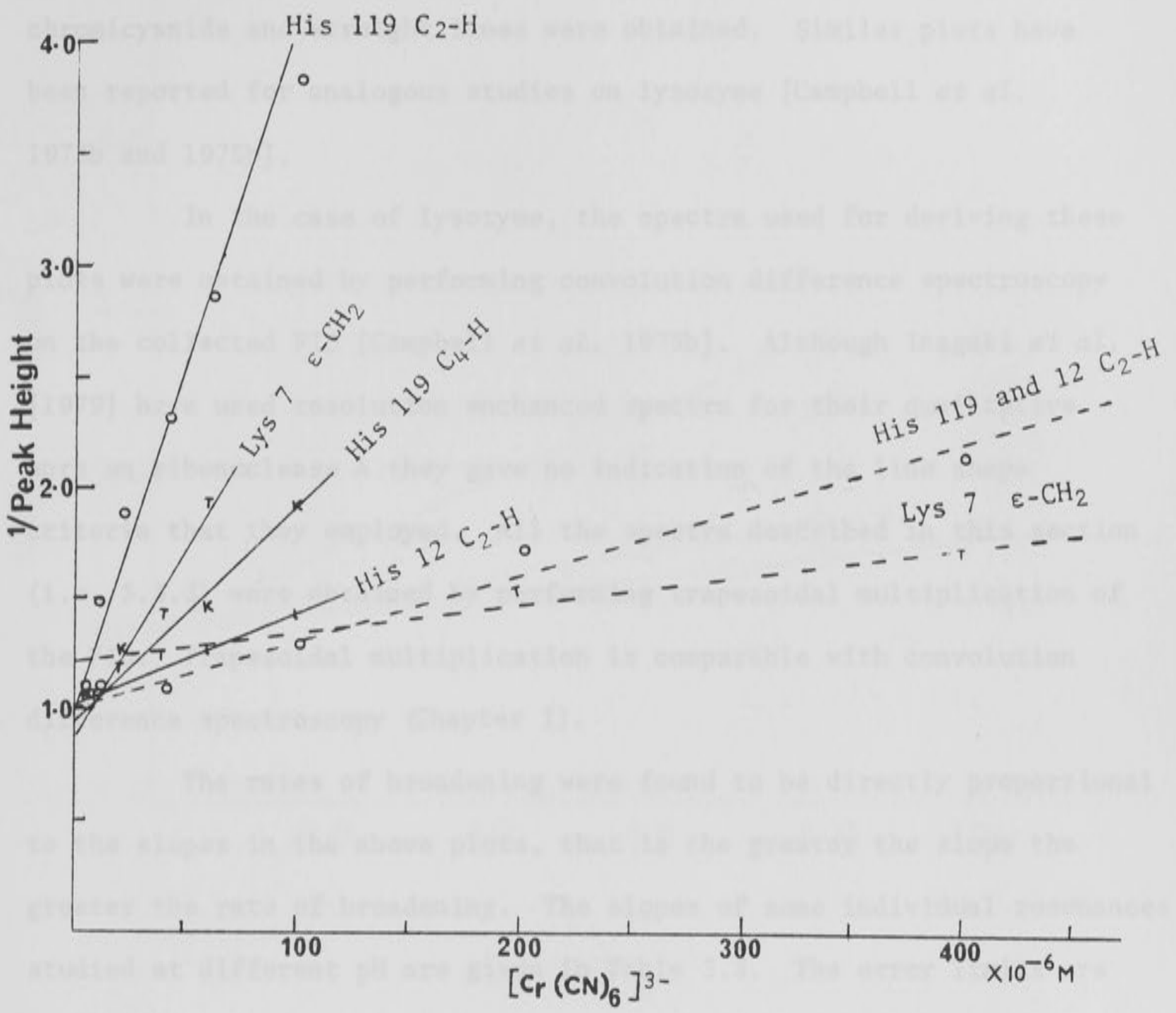


Figure 3.20 Rates of broadening of resonances as a function of chromicyanide addition to ribonuclease A in the presence and in the absence of the inhibitor at pH 5.3. Other experimental details in Figures 3.18 and 3.19.
(——) without inhibitor; (– –) with inhibitor.

intensity when chromicyanide was added. The reciprocal of the normalised peak heights were plotted against the concentration of the chromicyanide and straight lines were obtained. Similar plots have been reported for analogous studies on lysozyme [Campbell *et al.* 1973b and 1975b].

In the case of lysozyme, the spectra used for deriving these plots were obtained by performing convolution difference spectroscopy on the collected FID [Campbell *et al.* 1975b]. Although Inagaki *et al.* [1979] have used resolution enchanced spectra for their qualitative work on ribonuclease A they gave no indication of the line shape criteria that they employed. All the spectra described in this section (i.e. 3.3.3) were obtained by performing trapezoidal multiplication of the FID. Trapezoidal multiplication is comparable with convolution difference spectroscopy (Chapter I).

The rates of broadening were found to be directly proportional to the slopes in the above plots, that is the greater the slope the greater the rate of broadening. The slopes of some individual resonances studied at different pH are given in Table 3.3. The error limits are small (±500) in comparison to the magnitude of these arbitrary slope values. The main source of error in this experiment is in measuring the

TABLE 3.3

Slopes of resonances from plots in Figures 3.20 and 3.21

	Ribonuclease A				Ribonuclease A + 3'-UMP	
pH	His 119 C_2-H	His 119 C_4-H	His 12 C_2-H	Lys 7 ε-CH_2	His 119 and His 12 C_2-H	Lys 7 ε-CH_2
4.0	2.22×10^4	-	9.38×10^3*	5.68×10^3	-	-
5.3	3.24×10^4	1×10^4	4.17×10^3	1.69×10^4	3.16×10^3	1.35×10^3

* The value obtained from a similar plot for the His 12 C_2-H resonance using normal spectra from the same experiment was within 5% of the above mentioned value indicating the validity of the resolution enhancement method employed. The rate of broadening of only the His 12 C_2-H resonance was determined using the normal spectra as this resonance was distinctly observed from the others.

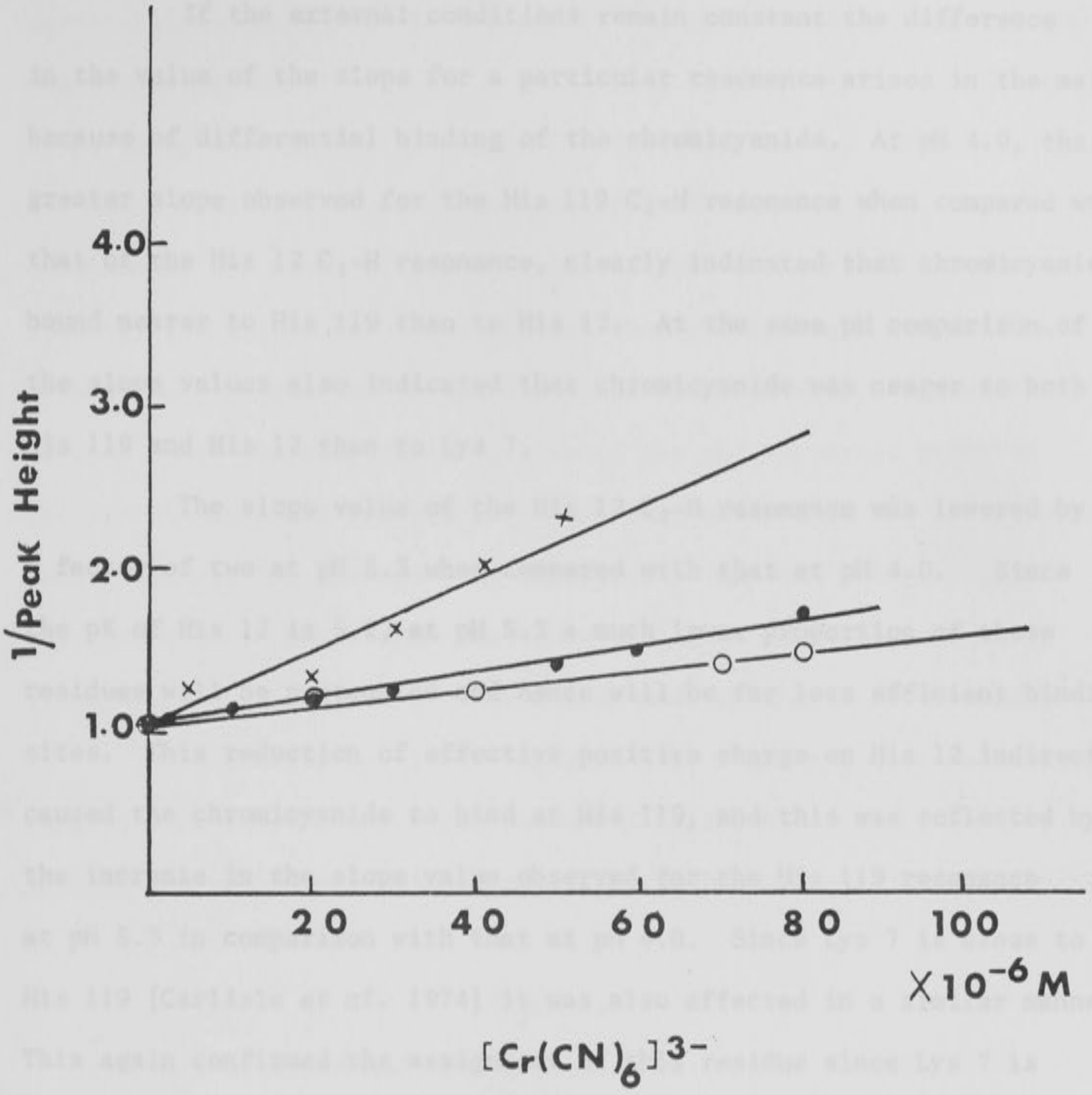


Figure 3.21 Rates of broadening of resonances of ribonuclease A at pH 4.0 as function of chromicyanide concentration. X - His 119 C_2-H; ●- His 12 C_2-H; O - Lys 7 ε-CH_2.

peak heights of the resonances. In the resolution enhanced (trapezioidal multiplication) spectra it is difficult to get a good approximation for the baseline of the individual peaks due to slight distortion of the line shapes. Although this could be overcome by using normal spectra, the latter has the disadvantage of revealing only one or two resonances distinctly.

If the external conditions remain constant the difference in the value of the slope for a particular resonance arises in the main because of differential binding of the chromicyanide. At pH 4.0, the greater slope observed for the His 119 C_2-H resonance when compared with that of the His 12 C_2-H resonance, clearly indicated that chromicyanide bound nearer to His 119 than to His 12. At the same pH comparison of the slope values also indicated that chromicyanide was nearer to both His 119 and His 12 than to Lys 7.

The slope value of the His 12 C_2-H resonance was lowered by a factor of two at pH 5.3 when compared with that at pH 4.0. Since the pK of His 12 is 5.2, at pH 5.3 a much lower proportion of these residues will be protonated and hence will be far less efficient binding sites. This reduction of effective positive charge on His 12 indirectly caused the chromicyanide to bind at His 119, and this was reflected by the increase in the slope value observed for the His 119 resonance at pH 5.3 in comparison with that at pH 4.0. Since Lys 7 is close to His 119 [Carlisle *et al.* 1974] it was also affected in a similar manner. This again confirmed the assignment of this residue since Lys 7 is closer to His 119 than to Lys 41. The slope values at this pH also indicated that the binding of chromicyanide was nearer the His 119 C_2-H atom than the C_4-H atom of the imidazole moiety. It can also be seen from Table 3.3 that inhibitor binding to ribonuclease A at pH 5.3 substantially reduces the effect of chromicyanide on these residues (<10 times).

The distances of various atoms or groups from the chromicyanide binding site can be calculated in arbitrary units from the observed slope values. Assuming that τ_c, the correlation time (3.3) is the same for all the protons in this study, the slope value for a

particular resonance can be related to $\frac{1}{T_2}$, the relaxation rate of the corresponding nuclei. If $f(\tau_c)$ is assumed to be unity then from the slope value some arbitrary values for distances can be obtained. If the exact binding site of chromicyanide were known from the three-dimensional structure of the protein molecule obtained from x-ray studies, the values for the distances obtained by the above mentioned method could be related to the distances obtained from x-ray studies. However since the exact binding site of the chromicyanide anion in the protein molecule remains uncertain and varies with the pH employed, such a correlation has not been attempted. Instead, the arbitrary distance values obtained in the above mentioned manner are given in Table 3.4 and quantify the observations made on the observed slopes.

In conclusion, it is apparent that a paramagnetic relaxing reagent like chromicyanide can be useful as a structural probe for mapping the positively charged active sites of enzymes like ribonuclease A.

TABLE 3.4

Distances of atoms and groups from chromicyanide binding site*

pH	Ribonuclease A				Ribonuclease A + 3'-UMP	
	His 119 C_2-H	His 119 C_4-H	His 12 C_2-H	Lys 7 ε-CH_2	His 119 and His 12 C_2-H	Lys 7 ε-CH_2
4.0	0.189	-	0.218	0.237	-	-
5.30	0.177	0.215	0.249	0.197	0.261	0.3

* Arbitrary units.

3.3.3.2 Potassium ferricyanide ($K_3Fe(CN)_6$)

The addition of potassium ferricyanide to ribonuclease A was studied at pH 4.0. Although the ferricyanide anion is paramagnetic

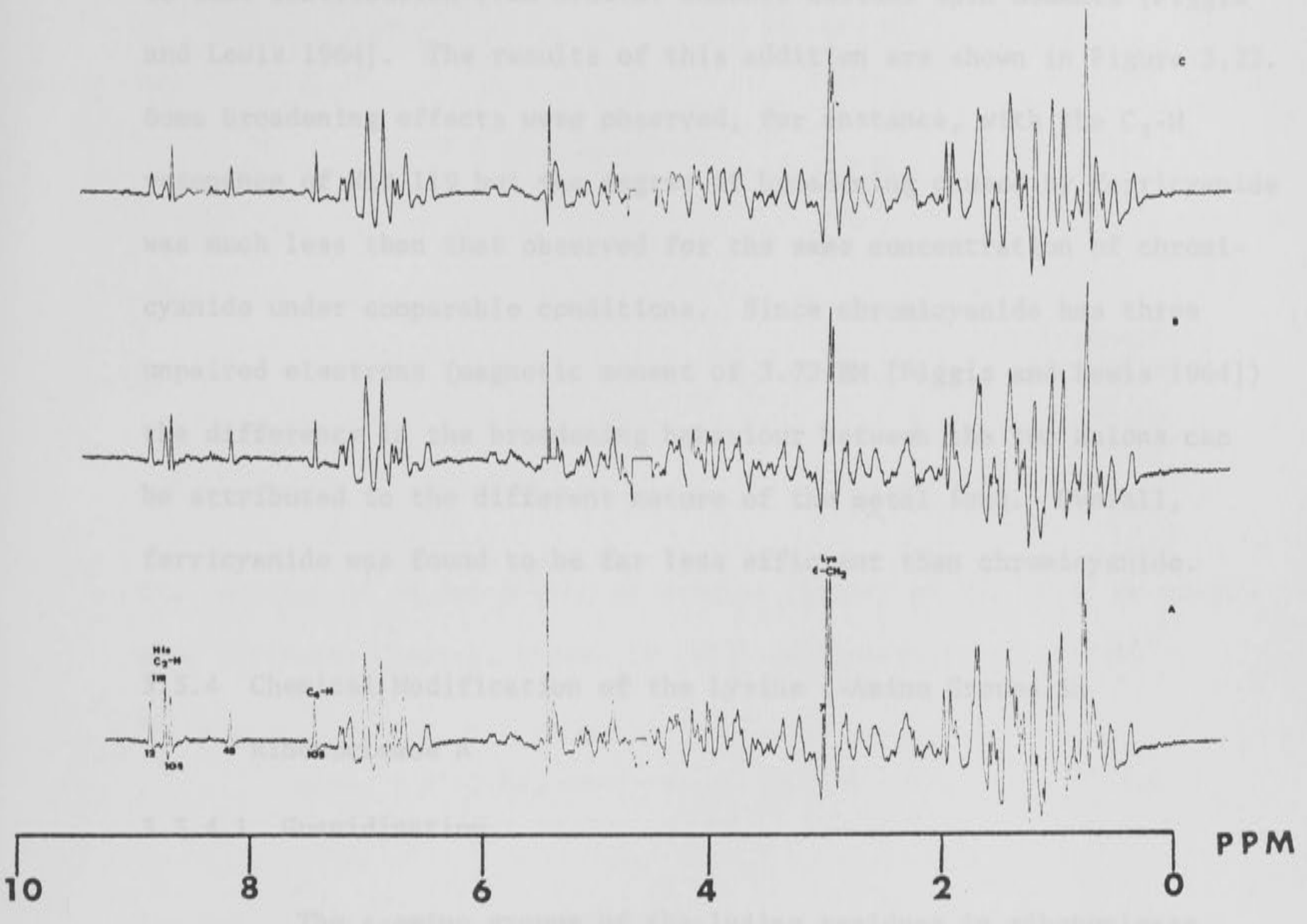


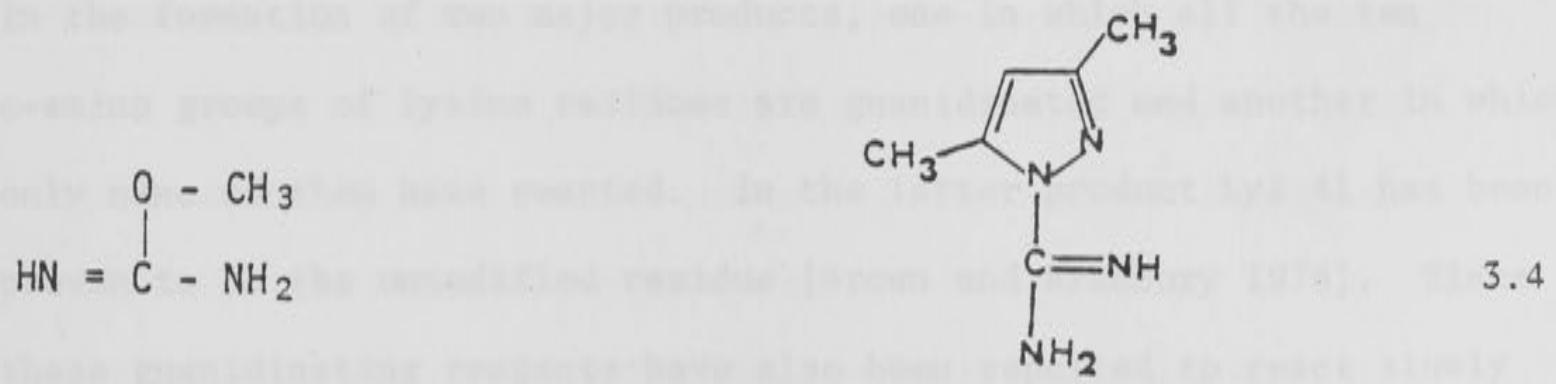
Figure 3.22 Effect of ferricyanide on ribonuclease A studied at 270 MHz. RNase A = 7.25 mM; NaCl = 0.3 M; pH = 4.0; Temperature = 35°C. 1 mM solution of potassium ferricyanide was used for these additions. A - 0 μM; B - 80 μM; C - 200 μM.

with one unpaired electron, the magnetic moment of 2.40 BM corresponds to some contribution from orbital moments besides spin moments [Figgis and Lewis 1964]. The results of this addition are shown in Figure 3.22. Some broadening effects were observed, for instance, with the C_2-H resonance of His 119 but the degree of broadening caused by ferricyanide was much less than that observed for the same concentration of chromicyanide under comparable conditions. Since chromicyanide has three unpaired electrons (magnetic moment of 3.72 BM [Figgis and Lewis 1964]) the difference in the broadening behaviour between the two anions can be attributed to the different nature of the metal ions. Overall, ferricyanide was found to be far less efficient than chromicyanide.

3.3.4 Chemical Modification of the Lysine ε-Amino Groups in Ribonuclease A

3.3.4.1 Guanidination

The ε-amino groups of the lysine residues in ribonuclease A can be guanidinated with either O-methylisourea or 1-guanyl-3,5-dimethylpyrazole (GDMP).

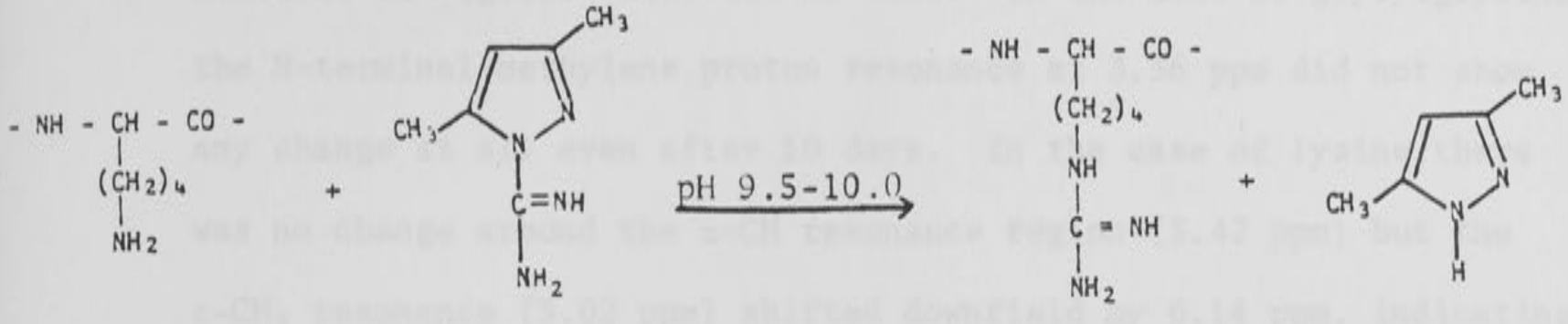


3.4

O-methylisourea

1-guanyl-3,5-dimethylpyrazole

The reaction between GDMP and lysine residues can be depicted as follows [Habeeb 1960].



3.5

lysine GDMP homoarginine 3,5-dimethyl-pyrazole

The reaction of an amine with an amidine [Shiner *et al.* 1944] or guanidine [Nitrogen Chemical Digest IV 1950] can generally be written as

$$R\text{-}NH_2 + R'\text{-}\underset{\underset{X}{\|}}{C}\text{-}NH_2 \longrightarrow R'\text{-}\underset{\underset{X}{\|}}{C}\text{-}NH\text{-}R + NH_3 \qquad 3.6$$

The reaction is generally thought to occur by an addition-elimination mechanism (or possibly, by a SN_2 type displacement [McKay 1952]).

With ribonuclease A at pH 10.0 guanidination with GDMP results in the formation of two major products, one in which all the ten ε-amino groups of lysine residues are guanidinated and another in which only nine of them have reacted. In the latter product Lys 41 has been proven to be the unmodified residue [Brown and Bradbury 1976]. Since these guanidinating reagents have also been reported to react slowly at the α-amino group of amino acids [Fusold *et al.* 1961], it was necessary to confirm that this reaction did not occur to a significant extent at the α-amino group of Lys 1 in ribonuclease A. Hence simple peptides and amino acids (e.g. Gly-Gly, Lys) were incubated with a slight excess of GDMP at pH 10.0 and the reaction was monitored

on the 100 MHz NMR instrument by obtaining spectra of these reaction mixtures at regular intervals of time. In the case of glycylglycine the N-terminal methylene proton resonance at 3.36 ppm did not show any change at all even after 10 days. In the case of lysine there was no change around the α-CH resonance region (3.42 ppm) but the ε-CH_2 resonance (3.02 ppm) shifted downfield by 0.14 ppm, indicating that guanidination had occurred at the ε-amino group (Figure 3.23).

The chromatogram for the separation of these guanidinated products are shown in Figure 3.24. A higher pH for the elution gradient (pH 6.6) than that used (pH 6.37) by Brown and Bradbury [1976] gave an improved yield (45%) of this product. Amino acid analyses were carried out on both the guanidinated products obtained, Table 3.1. When activity measurements were performed on the guanidinated products of ribonuclease A, the nonaguanidino derivative showed an activity of 75% that of the native enzyme, while that with the ten lysine residues guanidinated exhibited 15% activity (by the method of Kunitz [1946].

Due to the introduction of a guanidino group into the lysine residues of ribonuclease A, the derivatized enzyme will be more basic than the original (pK of arginine-guanidino group is 12.5). Hence a stronger deshielding effect will be experienced by the ε-CH_2 protons resulting in a downfield shift of the resonances. Thus a comparison of the pH titration series of the nona and decaguanidino derivatives should readily reveal the Lys 41 resonances in the nona-guanidino derivative. This argument is based upon the fact that guanidination does not change the three-dimensional structure of the molecule, at least that near the active site, as can be inferred from the activity of 75% observed for this derivative. However the pK of the ε-amino group of Lys 41 in the nonaguanidino derivative would

Figure 3.23 Reaction of GDMP with lysine studied at 80 MHz with a 5% soln. of the amino acid at pH 9.8 with an equi-molar amount of GDMP.

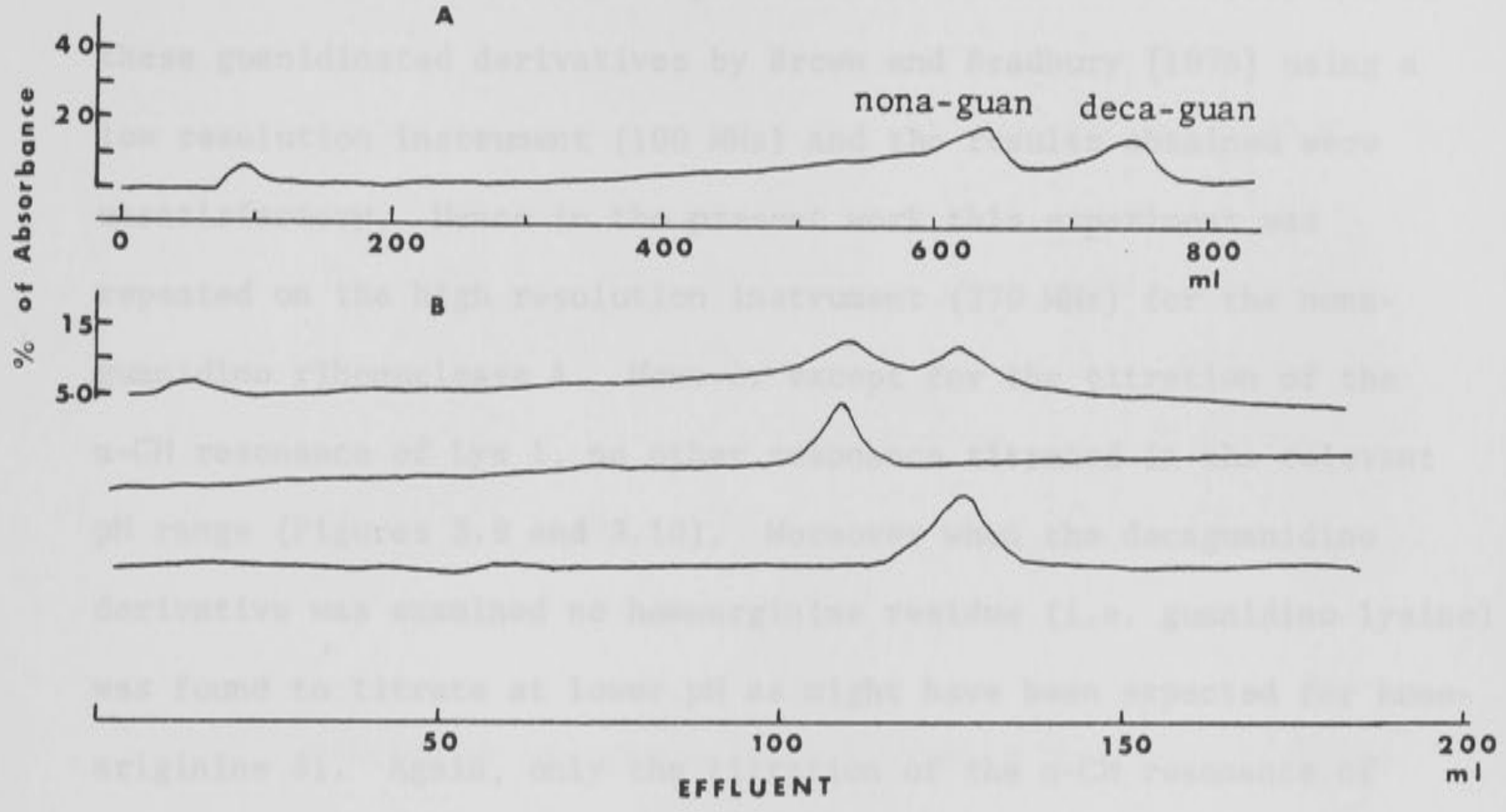


Figure 3.24 Chromatography of the guanidinated derivatives of ribonuclease A.
A - Preparatory column. 2.1 x 40 cm packed with Bio-Rex 70 (200-400 mesh). Elution gradient, 1.0 litre of 0.15 M PO_4^{3-} buffer to 1.0 litre of 0.4 M PO_4^{3-} buffer both at pH 6.6. Sample size, 300 mg. Eluting rate, 42 ml/hr. Fractions, 10 ml.
B - Analytical column. 0.6 x 40 cm packed with the above mentioned ion-exchange resin. Elution gradient, 70 ml of 0.15 M PO_4^{3-} buffer to 70 ml of 0.4 M PO_4^{3-} buffer at pH 6.6. Sample size, 10 mg. Eluting rate, 6 ml/hr.

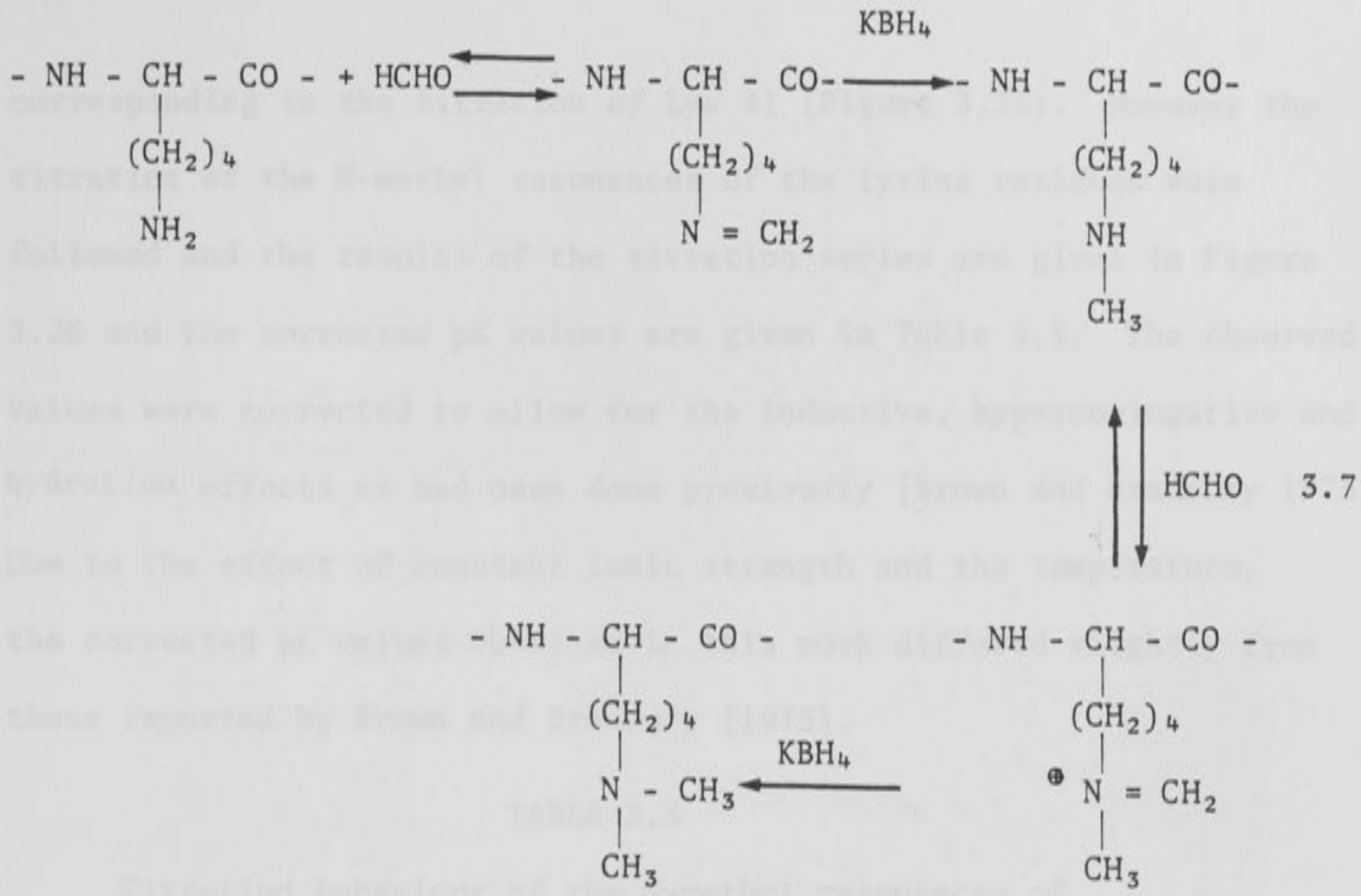
be expected to be less than 9.0 (as observed in the native enzyme) because of modification of the other lysine residues near the active site (i.e. Lys 7 and Lys 66) by guanidination.

With this in mind a pH titration series was carried out on these guanidinated derivatives by Brown and Bradbury [1976] using a low resolution instrument (100 MHz) and the results obtained were unsatisfactory. Hence in the present work this experiment was repeated on the high resolution instrument (270 MHz) for the nona-guanidino ribonuclease A. However except for the titration of the α-CH resonance of Lys 1, no other resonance titrated in the relevant pH range (Figures 3.9 and 3.10). Moreover when the decaguanidino derivative was examined no homoarginine residue (i.e. guanidino lysine) was found to titrate at lower pH as might have been expected for homo-ariginine 41. Again, only the titration of the α-CH resonance of Lys 1 was observed.

Spin decoupling experiments were also carried out on the nona-guanidino ribonuclease A by irradiating the δ-CH_2 protons of the homoarginine residues. However irradiation at *ca.* 1.8 ppm only resulted in the sharpening of the α-CH resonance of Lys 1.

3.3.4.2 Methylation

Methylation of proteins by the method of Means and Feeney [1968] involves the formation of a Schiff base between formaldehyde and the ε-amino group of the lysine residues and subsequent reduction with potassium borohydride. The reaction can be depicted as follows



Methylation of ribonuclease A by this procedure resulted in the formation of mainly dimethyllysine residues and also some monomethyllysine residues [Brown and Bradbury 1975]. An NMR study of methylated ribonuclease A was carried out by Brown and Bradbury [1975] and they reported the pK of 11 amino groups in the protein as indicated by pH titration series carried out at 18°C (in the absence of constant ionic strength). Since this methylation aided in 'seeing' the N-methyl resonance of Lys 41 it was expected that it might also show a shift of the ε-CH_2 resonance of Lys 41 in the titration series of this compound. Brown and Bradbury [1975] were unable to observe this signal in their study so this aspect was reinvesitgated in the present work.

Several pH titration series were carried out on methylated ribonuclease A at 35°C in the presence of 0.3 M NaCl. Due to the complexity of the spectrum around 3.0 ppm (due to the overlapping of the ε-CH_2 triplets of lysine residues), it was difficult to detect any movement

corresponding to the titration of Lys 41 (Figure 3.25). However the titration of the N-methyl resonances of the lysine residues were followed and the results of the titration series are given in Figure 3.26 and the corrected pK values are given in Table 3.5. The observed values were corrected to allow for the inductive, hyperconjugative and hydration effects as had been done previously [Brown and Bradbury 1975]. Due to the effect of constant ionic strength and the temperature, the corrected pK values obtained in this work differed slightly from those reported by Brown and Bradbury [1975].

TABLE 3.5

Titration behaviour of the N-methyl resonances of methylated ribonuclease A

Resonance	Residue	pK observed[a]	pK corrected[b]
1	Lys 1 (α-CH)	7.90	7.50
2	Lys 1 (N^{α}-CH_3)	7.49	7.09
3	Lys 41 (N^{ε}-CH_3)	9.27	8.97
4	Lys 7 (N^{ε}-CH_3)[c]	10.2	10.8
5		10.72	11.32
6		10.75	11.35
7		10.87	11.47
8		10.47	11.07
9		10.47	11.07
10		10.52	11.12
11		10.46	11.06
12		10.46	11.06

[a] pK values calculated from pH meter readings uncorrected for deuterium isotope effects. They were determined at 35°C in the presence of 0.3 M NaCl with 2.3 mM of the methylated derivative.

[b] The observed pK values were corrected for monomethylation or dimethylation as outlined by Brown and Bradbury [1975].

[c] Tentative assignment.

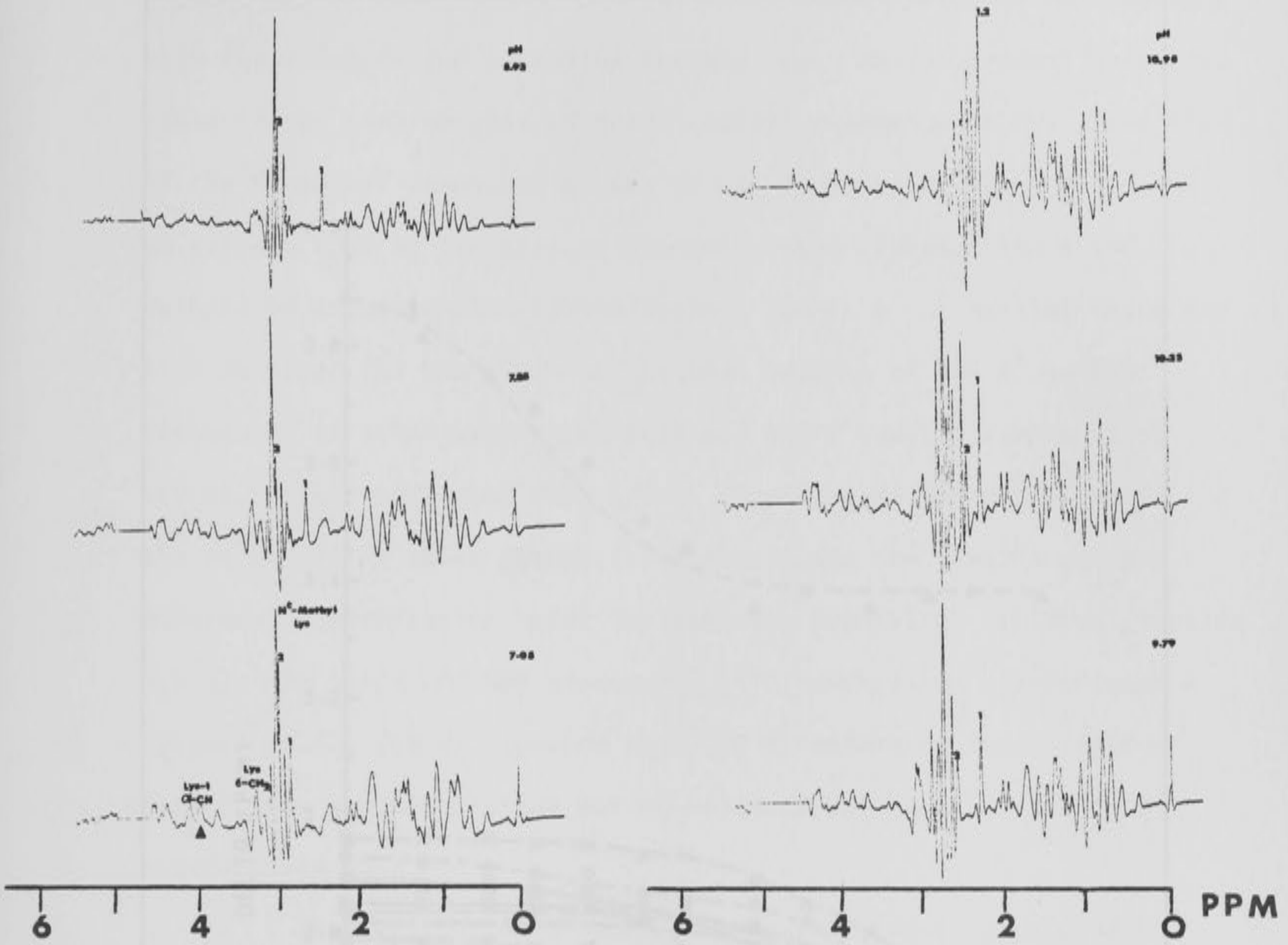


Figure 3.25 Titration of the methylated lysine residues of methylated ribonuclease A. The titration series was carried out with 3.62 mM of the protein solution containing 0.3 M NaCl at 35°C.

1 - Lys 1 N^{α}-dimethyl; 2 - Lys 41 N^{ε}-methyl.

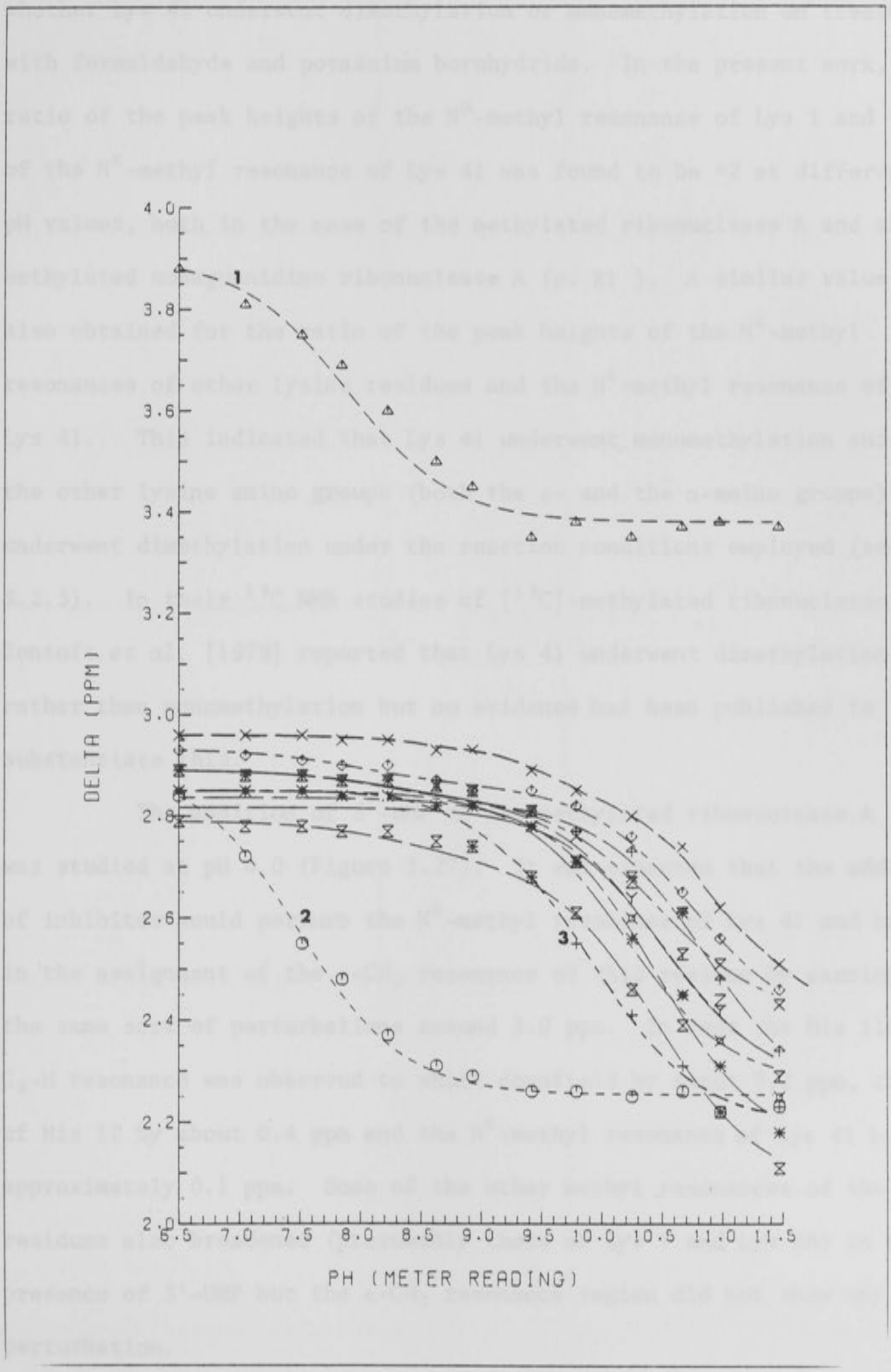


Figure 3.26 Titration curves for the methylated lysine residues of methylated ribonuclease A.

1 - Lys 1 α-CH; 2 - Lys 1 N^{α}- dimethyl; 3 - Lys 41 N^{ε}- methyl.

From the work of Brown and Bradbury [1975] it was not clear whether Lys 41 underwent dimethylation or monomethylation on treatment with formaldehyde and potassium borohydride. In the present work, the ratio of the peak heights of the N^{α}-methyl resonance of Lys 1 and that of the N^{ε}-methyl resonance of Lys 41 was found to be ≃2 at different pH values, both in the case of the methylated ribonuclease A and the methylated nonaguanidino ribonuclease A (p. 81). A similar value was also obtained for the ratio of the peak heights of the N^{ε}-methyl resonances of other lysine residues and the N^{ε}-methyl resonance of Lys 41. This indicated that Lys 41 underwent monomethylation and that the other lysine amino groups (both the ε- and the α-amino groups) underwent dimethylation under the reaction conditions employed (section 3.2.3). In their ^{13}C NMR studies of [^{13}C]-methylated ribonuclease A Jentoft *et al.* [1979] reported that Lys 41 underwent dimethylation rather than monomethylation but no evidence had been published to substantiate this.

The addition of 3'-UMP to the methylated ribonuclease A was studied at pH 6.0 (Figure 3.27). It was expected that the addition of inhibitor would perturb the N^{ε}-methyl resonance of Lys 41 and help in the assignment of the ε-CH_2 resonance of this residue by causing the same sort of perturbations around 3.0 ppm. In fact the His 119 C_2-H resonance was observed to shift downfield by about 0.2 ppm, that of His 12 by about 0.4 ppm and the N^{ε}-methyl resonance of Lys 41 by approximately 0.1 ppm. Some of the other methyl resonances of the lysine residues also broadened (presumably those of Lys 7 and Lys 66) in the presence of 3'-UMP but the ε-CH_2 resonance region did not show any perturbation.

The nonaguanidino ribonuclease A was also methylated for

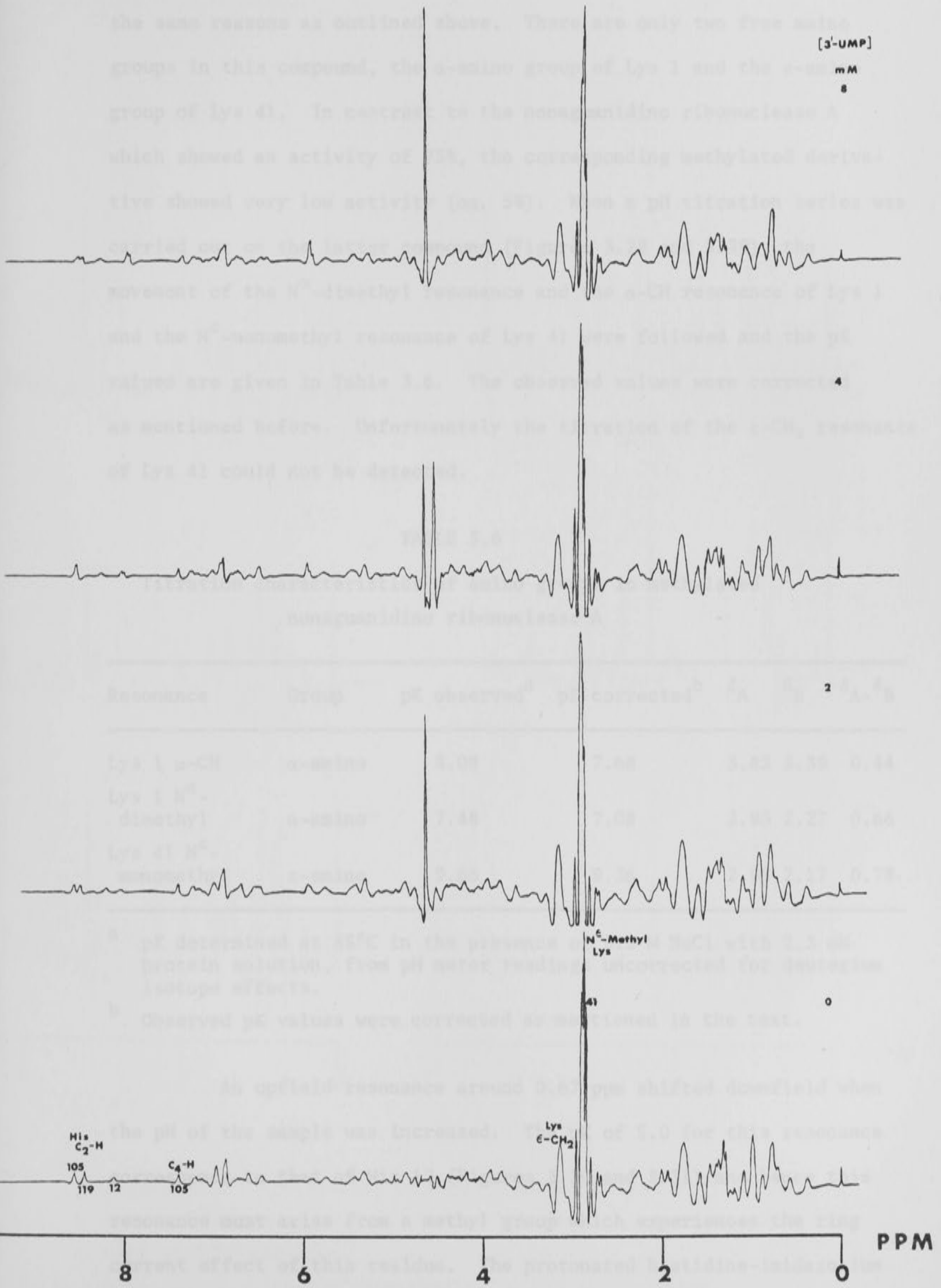


Figure 3.27 The effect of inhibitor (3'-UMP) addition to methylated ribonuclease A studied at 270 MHz. Conditions same

the same reasons as outlined above. There are only two free amino groups in this compound, the α-amino group of Lys 1 and the ε-amino group of Lys 41. In contrast to the nonaguanidino ribonuclease A which showed an activity of 75%, the corresponding methylated derivative showed very low activity (*ca.* 5%). When a pH titration series was carried out on the latter compound (Figures 3.28 and 3.29), the movement of the N^{α}-dimethyl resonance and the α-CH resonance of Lys 1 and the N^{ε}-monomethyl resonance of Lys 41 were followed and the pK values are given in Table 3.6. The observed values were corrected as mentioned before. Unfortunately the titration of the ε-CH_2 resonance of Lys 41 could not be detected.

TABLE 3.6

Titration characteristics of amino groups in methylated nonaguanidino ribonuclease A

Resonance	Group	pK observed[a]	pK corrected[b]	δ_A	δ_B	$\delta_A-\delta_B$
Lys 1 α-CH	α-amino	8.08	7.68	3.83	3.39	0.44
Lys 1 N^{α}-dimethyl	α-amino	7.48	7.08	2.93	2.27	0.66
Lys 41 N^{ε}-monomethyl	ε-amino	9.66	9.36	2.95	2.17	0.78

[a] pK determined at 35°C in the presence of 0.3 M NaCl with 2.3 mM protein solution, from pH meter readings uncorrected for deuterium isotope effects.

[b] Observed pK values were corrected as mentioned in the text.

An upfield resonance around 0.67 ppm shifted downfield when the pH of the sample was increased. The pK of 5.0 for this resonance corresponds to that of His 12 (Figures 3.30 and 3.31) and hence this resonance must arise from a methyl group which experiences the ring current effect of this residue. The protonated histidine-imidazolium

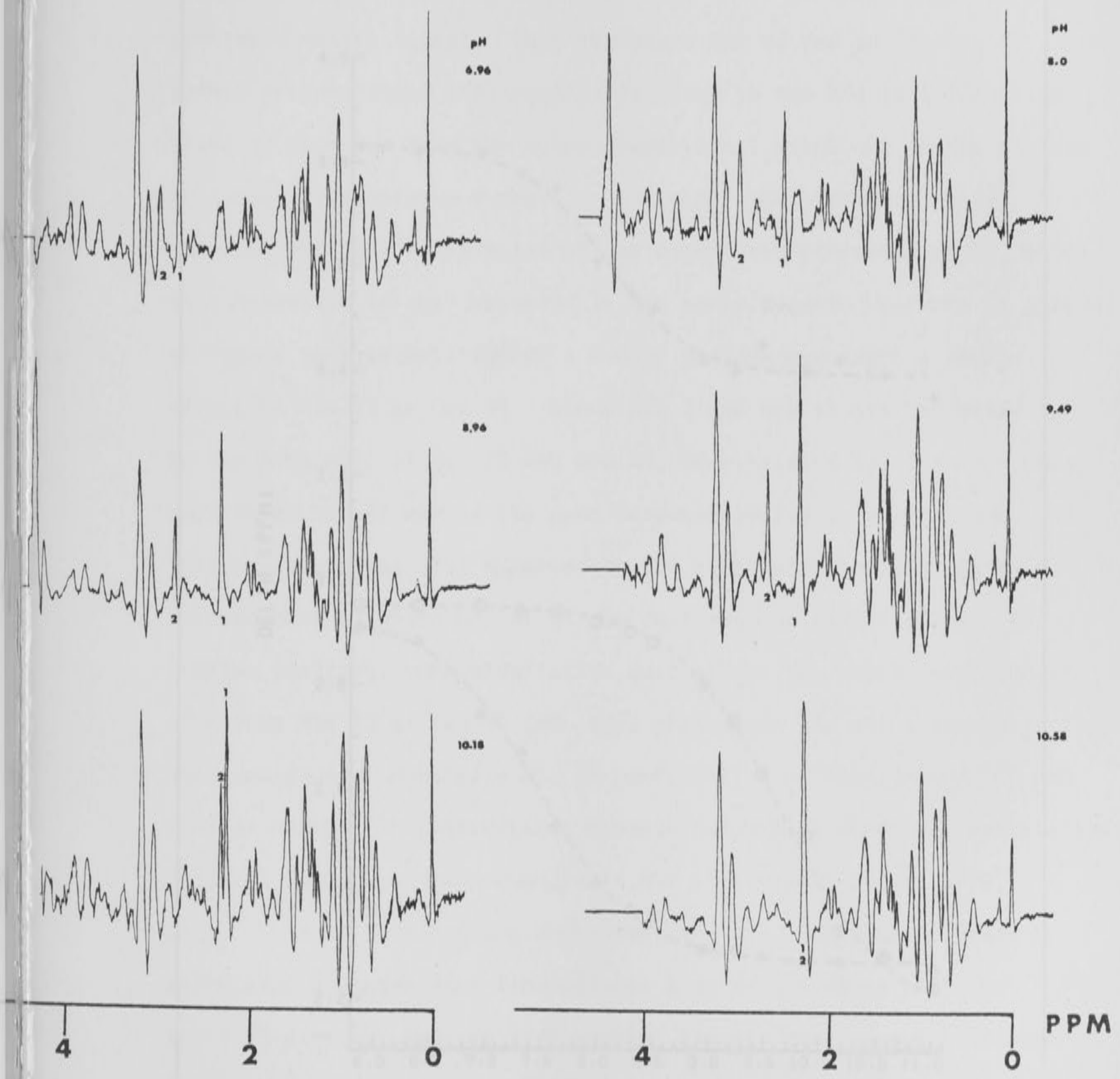


Figure 3.28 The spectra of methylated nonaguanidino ribonuclease A as a function of pH. 1.45 mM of the protein solution with 0.3 M NaCl at 35°C was used.

1 - Lys 1 N^{α}-dimethyl; 2 - Lys 41 N^{ε}-methyl.

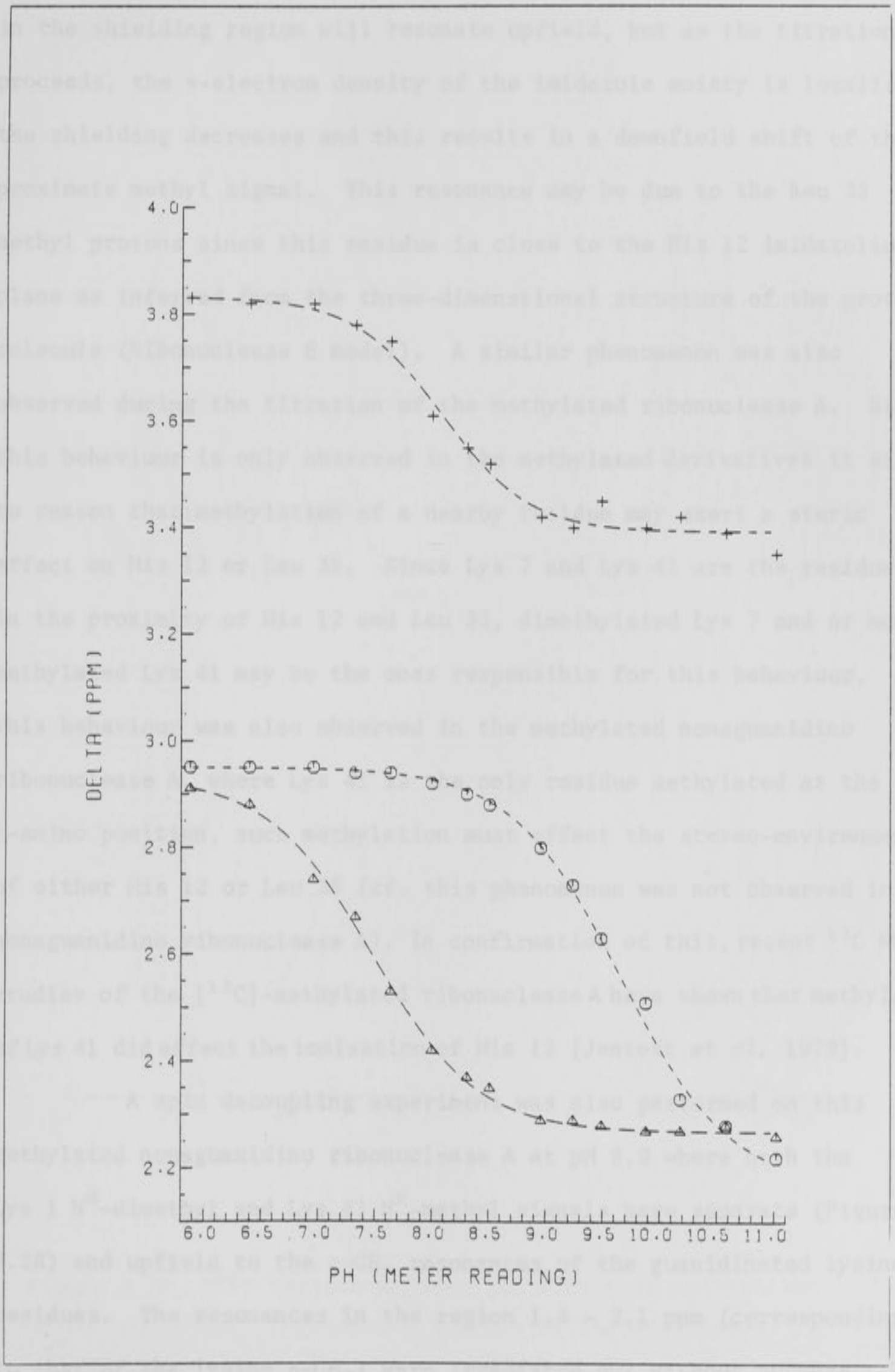


Figure 3.29 Titration curves for the methylated lysine residues of methylated nonaguanidino ribonuclease A.
+ - Lys 1 α-CH; O - Lys 41 N^{ε}-methyl; Δ - Lys 1 N^{α}-dimethyl.

form will possess a significant ring current and a methyl group in the shielding region will resonate upfield, but as the titration proceeds, the π-electron density of the imidazole moiety is localised, the shielding decreases and this results in a downfield shift of the proximate methyl signal. This resonance may be due to the Leu 35 methyl protons since this residue is close to the His 12 imidazolium plane as inferred from the three-dimenstional structure of the protein molecule (Ribonuclease S model). A similar phenomenon was also observed during the titration of the methylated ribonuclease A. Since this behaviour is only observed in the methylated derivatives it stands to reason that methylation of a nearby residue may exert a steric effect on His 12 or Leu 35. Since Lys 7 and Lys 41 are the residues in the proximity of His 12 and Leu 35, dimethylated Lys 7 and or mono-methylated Lys 41 may be the ones responsible for this behaviour. As this behaviour was also observed in the methylated nonaguanidino ribonuclease A, where Lys 41 is the only residue methylated at the ε-amino position, such methylation must affect the stereo-environment of either His 12 or Leu 35 (cf. this phenomenon was not observed in the nonaguanidino ribonuclease A). In confirmation of this, recent ^{13}C NMR studies of the [^{13}C]-methylated ribonuclease A have shown that methylation of Lys 41 did affect the ionisation of His 12 [Jentoft *et al.* 1979].

A spin decoupling experiment was also performed on this methylated nonaguanidino ribonuclease A at pH 8.9 where both the Lys 1 N^{α}-dimethyl and Lys 41 N^{ε}-methyl signals were separate (Figure 3.28) and upfield to the ε-CH_2 resonances of the guanidinated lysine residues. The resonances in the region 1.4 - 2.1 ppm (corresponding to that of the lysine δ-CH_2) were irradiated but without success. The ε-CH_2 resonance region did not show any perturbation that could be attributed to the resonances of Lys 41.

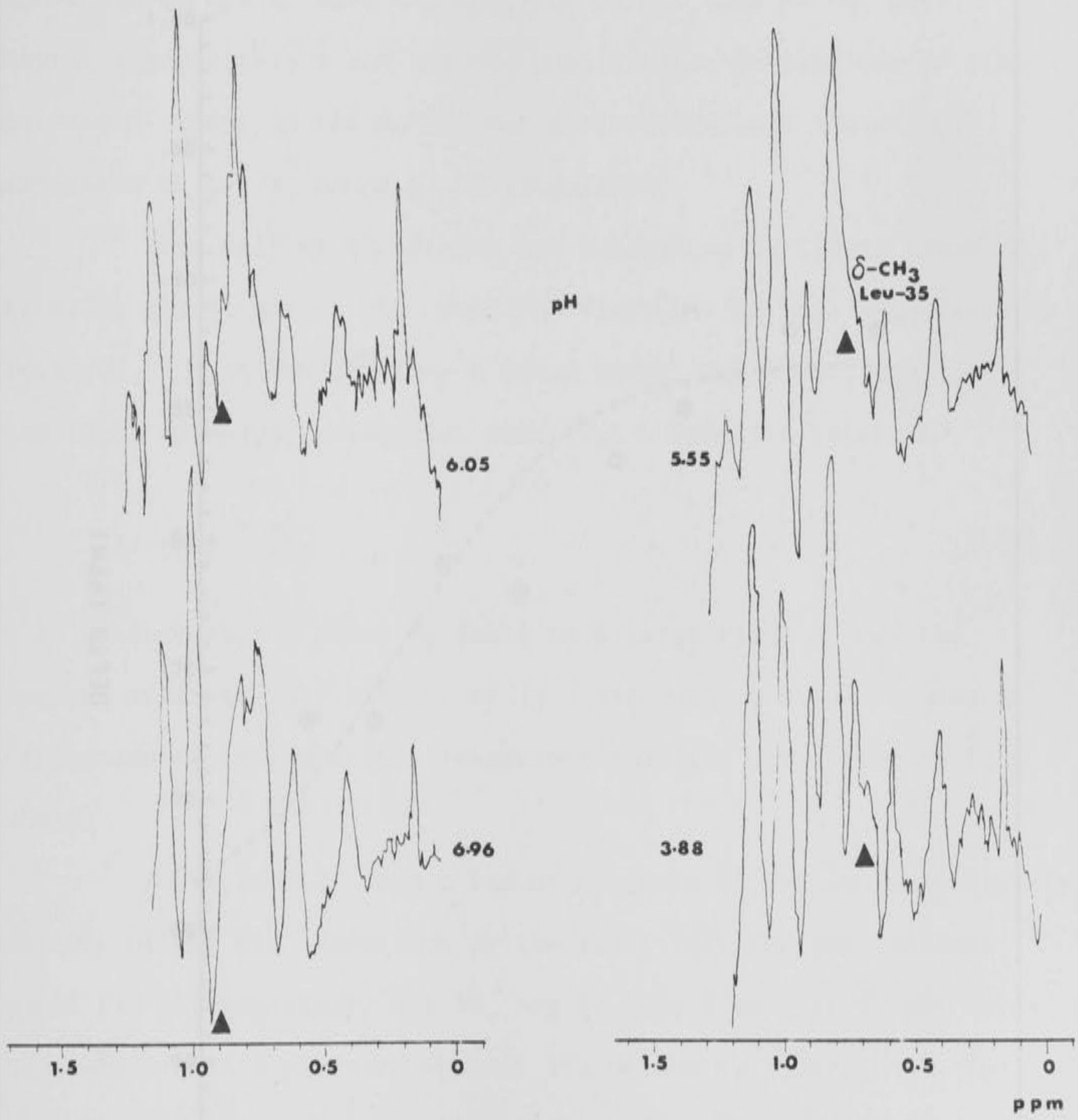


Figure 3.30 The spectra showing the effect of methylation of Lys 41 on Leu 35 methyl resonance (δ-CH_3) in the methylated nonaguanidino ribonuclease A at different pH.

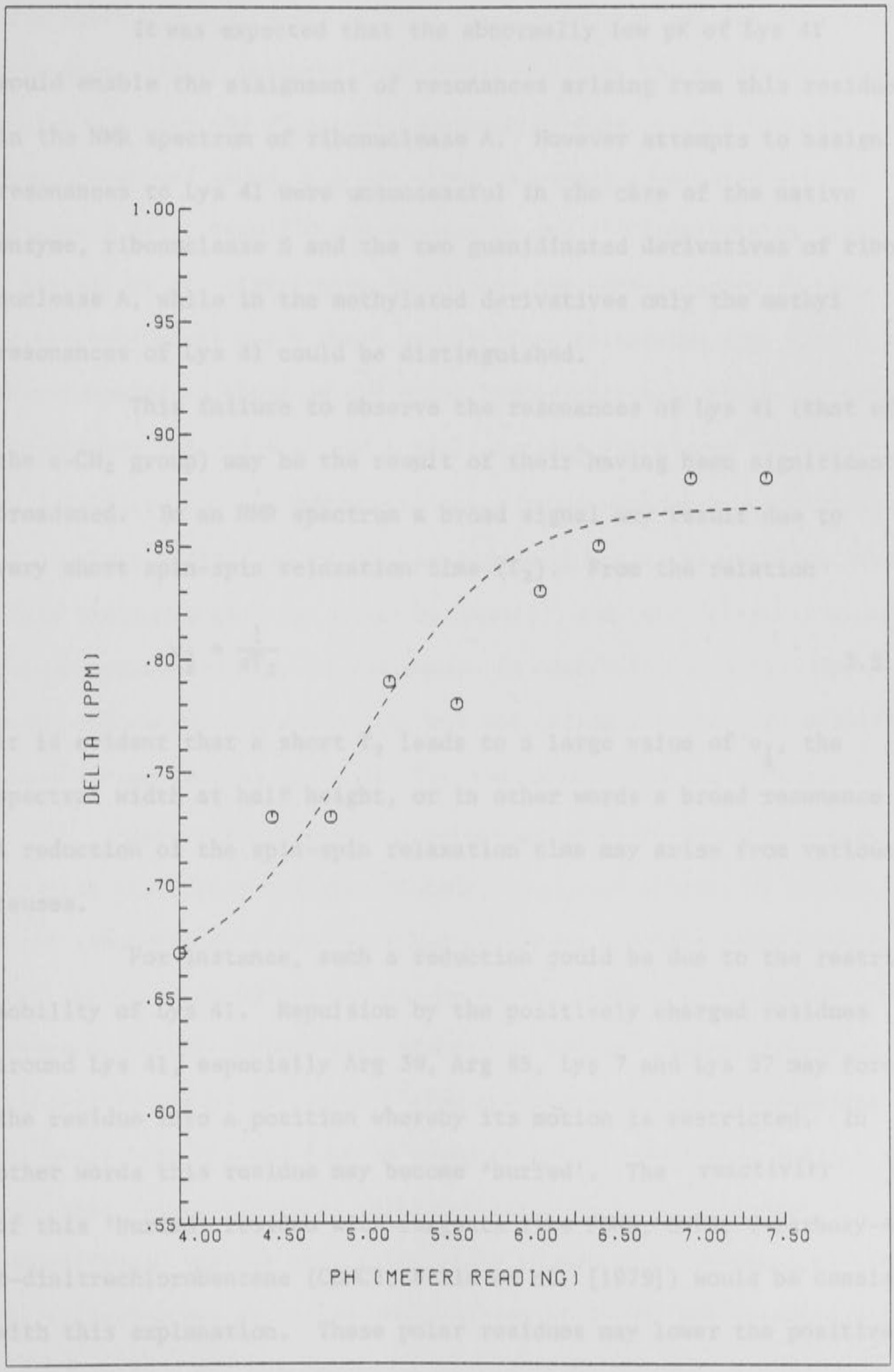


Figure 3.31 Methylated nonaguanidino ribonuclease A: Perturbation of the Leu 35 methyl group.

3.4 LYSINE 41

It was expected that the abnormally low pK of Lys 41 would enable the assignment of resonances arising from this residue in the NMR spectrum of ribonuclease A. However attempts to assign resonances to Lys 41 were unsuccessful in the case of the native enzyme, ribonuclease S and the two guanidinated derivatives of ribonuclease A, while in the methylated derivatives only the methyl resonances of Lys 41 could be distinguished.

This failure to observe the resonances of Lys 41 (that of the ε-CH_2 group) may be the result of their having been significantly broadened. In an NMR spectrum a broad signal may result due to very short spin-spin relaxation time (T_2). From the relation

$$\nu_{\frac{1}{2}} = \frac{1}{\pi T_2} \qquad 3.8$$

it is evident that a short T_2 leads to a large value of $\nu_{\frac{1}{2}}$, the spectral width at half height, or in other words a broad resonance. A reduction of the spin-spin relaxation time may arise from various causes.

For instance, such a reduction could be due to the restricted mobility of Lys 41. Repulsion by the positively charged residues around Lys 41, especially Arg 39, Arg 85, Lys 7 and Lys 37 may force the residue into a position whereby its motion is restricted. In other words this residue may become 'buried'. The reactivity of this 'buried' residue with reagents like FDNB, SNFB, 2-carboxy-4, 6-dinitrochlorobenzene (CDNCB, Bello *et al.* [1979]) would be consistent with this explanation. These polar residues may lower the positive character of the active site, which may release this molecule from a 'frozen' position and hence facilitate the reaction. It can be

mentioned here that guanidinating reagents like GDMP and O-methylisourea which are positively charged react with this residue less readily even under highly favourable pH conditions. In the methylated ribonuclease A only the N^{ε}-methyl resonance of this residue was affected when 3'-UMP was added, not any other resonance associated with it (Figure 3.27). From ^{13}C NMR studies of the [^{13}C]-methylated ribonuclease A Jentoft *et al.* [1981] have found that the methyl carbon of the methylated Lys 41 has a short spin-lattice relaxation time (T_1, 0.4 s when compared to those of the other lysine residues, whose T_1 values are between 0.8 - 0.9 s) and a small nuclear Overhauser enhancement (2.3 against 3.0 observed for the other lysine residues), implying that Lys 41 is in a relatively restricted environment. These observations substantiate the notion that Lys 41 may be 'buried', and hence possibly account for the apparent broad ε-CH_2 resonance. In contrast to this the other lysine ε-CH_2 resonances are sharp triplets indicating considerable mobility of these groups leading to longer T_2s and hence sharp resonances.

Restricted motion and accompanying short relaxation times may also arise from hydrogen bonding, but x-ray studies of ribonuclease A and ribonuclease S have revealed so far no hydrogen bonds between the ε-amino group of Lys 41 and any nearby residue (or residues). The existence of salt-bridge like structures between the negatively charged carboxylate groups of aspartic acid and glutamic acid residues and the positively charged ε-amino group of Lys 41 could also shorten the spin-spin relaxation time. Although metal ions and inhibitors can competitively inhibit this salt-bridge formation by interacting with the charged atoms or groups the addition of 3'-UMP and metal ions to ribonuclease A did not improve the resolution of ε-CH_2 protons of Lys 41 (section 3.3.2 and Chapters 4 and 5) so this appears an unlikely explanation.

3.5 CONCLUSIONS

The temperature dependence of the dissociation of the α-amino group of lysine 1 of ribonuclease A was studied by monitoring the α-CH resonance of this residue in the ^1H NMR spectrum. As a result the β-CH_2 resonance of lysine 1 could be assigned and its titration was studied. The assignment of this resonance was also confirmed by the spin decoupling experiments. Similar results were obtained with ribonuclease S, but all efforts to segregate the signals arising from the Lys 41 residue in both ribonuclease A and ribonuclease S failed.

An NMR study of the addition of inhibitors such as chloride ion, phosphate ion and 3'-UMP to ribonuclease A clearly indicated that they bound to the active site quite readily. Although several residues were affected when these inhibitors were added, the most significant feature observed was the differential behaviour of the histidines in the active site (i.e. His 12, His 119) as compared with the exposed histidines (His 105). 3'-UMP was found to affect the active site lysine residues (i.e. Lys 7 and Lys 66).

The binding of chromicyanide anion to ribonuclease A has also been studied and this anion is shown to bind closer to His 119 than His 12 thus indicating that the N1 nitrogen of His 119 can possibly be the binding site for this complex anion. The ε-CH_2 resonance of Lys 7 could be assigned given the position of this residue in the active site and its proximity to His 119. Several resonances (in the aliphatic end of ribonuclease A spectrum) arising from the residues in and around the active site have been tentatively assigned from this study. The rate of broadening of some of the resonances in the presence of chromicyanide was determined and from

this the relative distances of the respective groups from chromicyanide binding site were calculated. Hence it was apparent that a paramagnetic relaxing agent like chromicyanide could be useful as a structural probe for mapping the positively charged active sites of enzymes like ribonuclease A.

In both the nona and decaguanidino ribonuclease A the titration of the α-amino group of Lys 1 was detected. With the methylated derivatives of ribonuclease A (methylated ribonuclease A and the methylated nonaguanidino ribonuclease A) the titration of the N-methyl resonances of Lys 1 and Lys 41 were followed. Further the δ-CH_3 proton signal of Leu 35 was assigned and its proximity to His 12 was inferred from the observed pH dependent chemical shift.

However all attempts to assign resonances to Lys 41 (especially to the ε-CH_2 protons) in the NMR spectrum of ribonuclease A and its derivatives were unsuccessful.

CHAPTER 4

^{1}H NMR STUDIES ON LANTHANIDE BINDING TO RIBONUCLEASE A

4.1 INTRODUCTION

The use of lanthanides for studies of proteins by NMR methods have been extensively discussed [Nieboer 1975; Reuban 1975; Dobson and Levine 1976; Morris and Dwek 1977]. Lanthanides have been used for numerous studies of structure, NMR signal assignments and molecular dynamics [Inagaki and Miyazawa 1981]. While some of the lanthanides enhance the relaxation of bound or nearby nuclei (e.g. Gd^{3+}), most of them induce chemical shifts either by through-bond contact coupling or through space pseudo-contact coupling.*

For the paramagnetic relaxation reagents like Gd^{3+}, the paramagnetic contribution to relaxation time is given from the Solomon-Bloembergen equation as

$$T_2^{-1} = f(\tau_c) \cdot r^{-6} \qquad 4.1$$

where T_2^{-1} is the overall transverse relaxation rate, r is the lanthanide-metal distance and $f(\tau_c)$ is a complex function involving the correlation time τ_c for the dipolar interaction. A broadening reagent like Gd^{3+} can reveal resonances differentially at successive distances from the broadening probe binding position. Under the conditions of fast

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* Details in Chapter 1.

exchange, the amount of broadening can be controlled by the added Gd^{3+} concentration since the broadening $\propto [Gd^{3+}]/r^6$. Hence it is possible to see separately the spherical elements of the volume of space at greater and greater distances from the metal-containing protein molecule [Campbell *et al.* 1973b].

There are two general approaches to the study of proteins by using lanthanides. Firstly the protein itself can have one or several binding sites for the lanthanides. Lysozyme has a specific binding site for lanthanides and use has been made of this in assigning individual resonances and for studying the structure and dynamic properties of the protein in solution [Campbell *et al.* 1975; Dobson and Williams 1977]. Similar information has also been obtained by using lanthanide shift reagents in the study of bovine pancreatic trypsin inhibitor (BPTI, Perkins and Wüthrich [1978]). Alternatively, the protein can be chemically modified in order to chelate the lanthanides strongly at specific sites. In the case of BPTI tyrosine rings have been nitrated in order to selectively chelate the lanthanides [Marinetti *et al.* 1975-77].

Ribonuclease A has eleven free carboxyl groups which can bind the lanthanide metal ions, and these originate from aspartic and glutamic acid residues as well as the C-terminal valine residue. These include Asp 14, 38, 53, 83, 121; Glu 2, 9, 49, 86 and 111, in addition to Val 124. The binding of the lanthanide ions to such carboxyl groups is generally weak and based upon the studies with BPTI [Perkins and Wüthrich 1978], their binding constants can be expected to be of the order of 10-30 M^{-1}. All the carboxyl groups will compete for the added lanthanide metal ions and hence cannot give conclusive specific

information about the three-dimensional structure. This necessitates the introduction of strong binding sites for the lanthanides at specific regions in the protein molecule.

Such a study can be expected to provide more information in the case of a polypeptide made up of 20-30 amino acid residues where there are fewer structural complications. The employment of such a technique to the problem of macromolecules of molecular weight ≥10000 may not be as fruitful because of the various limitations involved. The major limitation is the structure of the macromolecule itself, the complex and compact nature of which causes complications with known physical methods. The use of a lanthanide relaxation reagent like Gd^{3+} will affect all the residues that are within its sphere of influence and hence is not specific or selective in its application. On the other hand, the pseudo-contact shifts of Eu^{3+}, Yb^{3+} are angularly dependent in the sense that they will affect only those residues that have some specific orientation with respect to the metal ions. These limitations arise due to the inherent nature of the lanthanide metal ions. The other limitations are chemical in nature. The ligand* for binding the lanthanides should be introduced specifically into the protein molecule at positions suitable for a convincing study and the lanthanides should bind more strongly to this ligand group than the free carboxyl groups in the protein molecule. Hence a suitable ligand for binding lanthanides should satisfy these criteria. Consequently the N-terminus of a protein may not be the best position to introduce these groups but for a polypeptide or a protein containing 20-30 amino acid residues the introduction of such a group at the N-terminus could well be more fruitful. Unfortunately in a protein like ribonuclease

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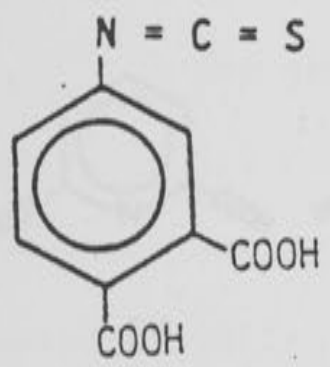
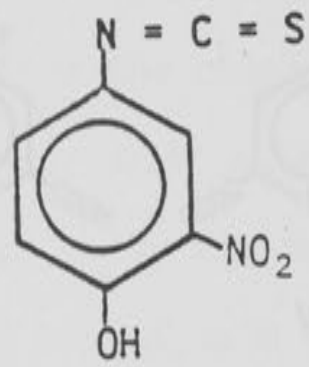
* The ligand refers to the introduced ones rather than the aspartic acid and glutamic acid residues in the protein molecule.

A, there are not many other positions which lend themselves to selective modification by a group capable of binding the lanthanides.

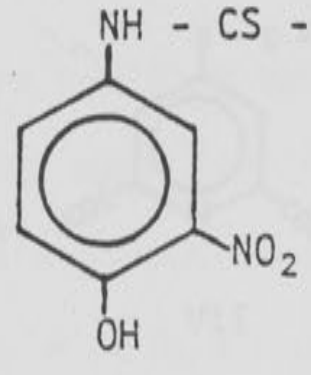
Previous attempts have utilized the reaction of ribonuclease A with 4-IPA (I, Bradbury *et al.* [1978]), 4-isothiocyanato-2-nitrophenol (II, Galloway [1978]) and the corresponding β-aminoethanol derivative (III). The binding constants of lanthanides with both compound I and III have been determined [Galloway 1978, Bradbury *et al.* 1980 unpublished results].



I



II



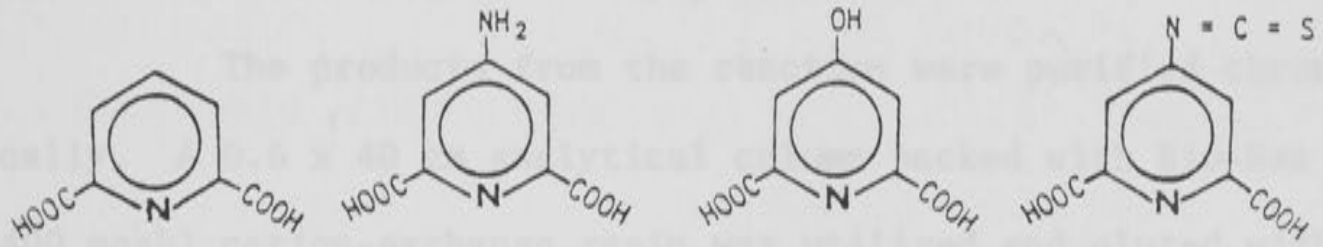
III

4.2

In order to increase the solubility of compound II, compound III was prepared by reacting compound II with β-aminoethanol. However whereas compound I and La^{3+} exhibited a binding constant of 220 M^{-1} (mono complex), compound III exhibited much weaker binding with a binding constant of 35 M^{-1} (comparable to those of the free carboxyl groups in the protein molecule).

Problems have previously arisen because the reagents (I and II) gave impure products with ribonuclease A, so considerable effort has now been made to purify those products, especially those obtained by the reaction of 4-IPA(I) with ribonuclease A (since 4-IPA-La^{3+} complex exhibits a high binding constant.) In order to accentuate these effects

attempts were also made to develop and introduce an alternative moiety at the N-terminus of ribonuclease A that binds the lanthanides far more strongly. It is known that 2,6-pyridinedicarboxylic acid (DPA) and its derivatives bind the lanthanides very strongly (the log K values for the mono complexes being of the order of 7-10 [Sillén and Martell 1964]). These derivatives have been found to behave as tridentate ligands with the lanthanides and form tris complexes where the rare earth metal ions exhibit a coordination number of nine



IV V VI VII 4.3

[Grenthe 1961]. The structure of these complexes in solution have been studied thoroughly by Alsaadi *et al.* [1980a and b].

Introduction of these ligands on the protein molecule can be expected to provide information about the structure of the protein molecule, at least that near the binding site. In an effort to utilise the chelating properties of the dipicolinic acid moiety attempts to prepare 4-isothiocyanato-2,6-pyridinedicarboxylic acid (4-IDPA, VII) were undertaken. A reagent such as VII should be effective in introducing a strong binding site for the lanthanides at the N-terminus of ribonuclease A. The reaction of the isothiocyanate (VII) with the peptide, Gly-Gly-Gly was also investigated as a preliminary model reaction.

4.2 EXPERIMENTAL

4.2.1 Reaction of 4-Isothiocyanatophthalic Acid with Ribonuclease A (Preparation of 4-IPA-RNase A)

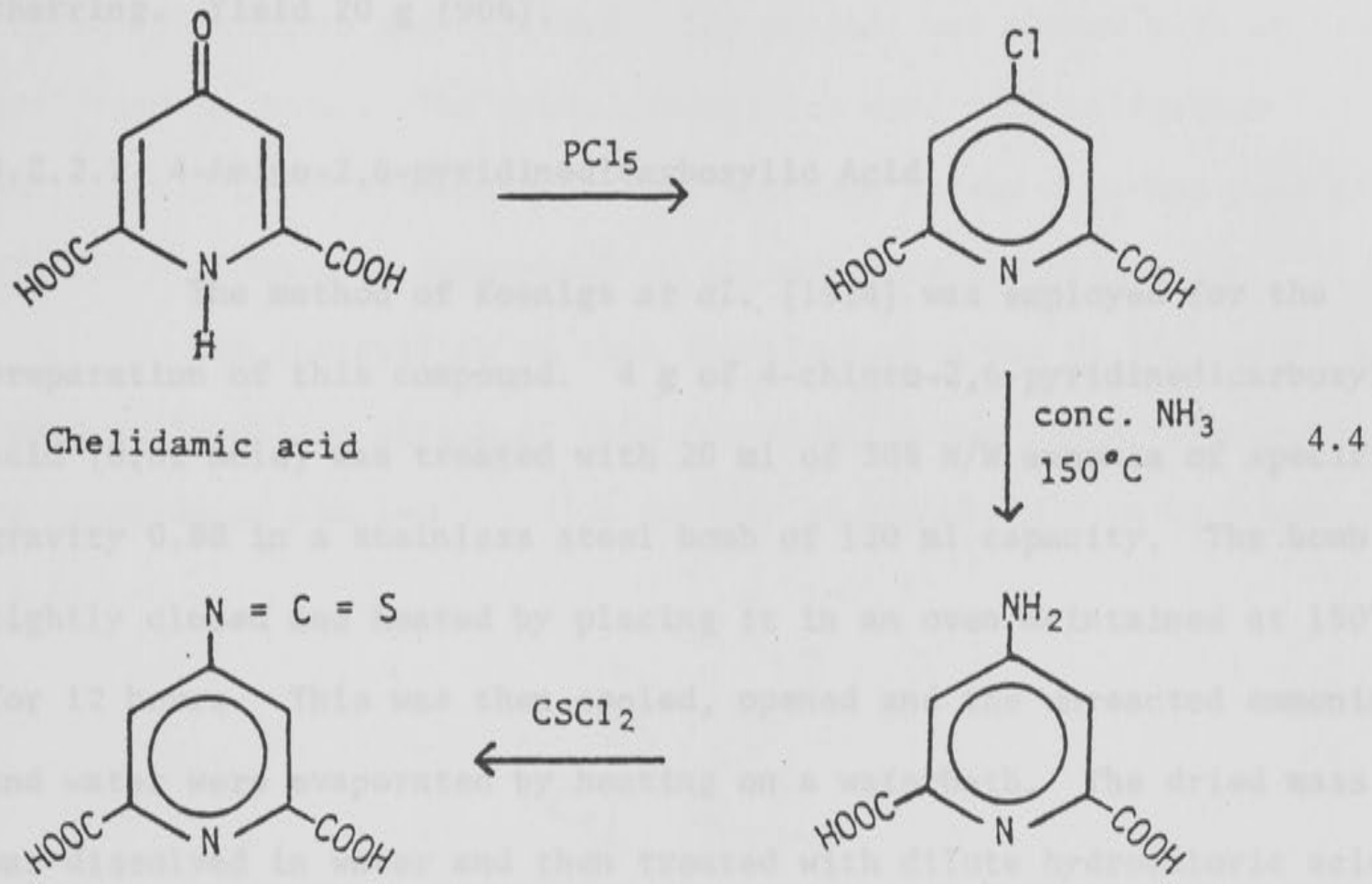
200 mg of ribonuclease A (14.4 micro moles) was dissolved in 10 ml of 0.1 M phosphate buffer, pH 7.2 and 8 mg of 4-isothiocyanatophthalic acid (35.8 micro moles) was added to this solution. The pH was adjusted to 7.2 and the reaction mixture was stirred at 25°C for 24 hours. After this time the reaction mixture was dialyzed extensively against distilled water and lyophilized.

The products from the reaction were purified chromatographically. A 0.6 x 40 cm analytical column packed with Bio-Rex 70 (200-400 mesh) cation-exchange resin was utilized and eluted with 0.2 M phosphate buffer, pH 6.0 at the rate of 6 ml/hr. This buffer was also used for equilibrating the column prior to addition of the reaction mixture (10 mg).

A preparatory column was set up for the bulk separation. A 1.2 x 100 cm column packed with the above mentioned ion-exchange resin was used. The column was eluted with 0.2 M phosphate buffer, pH 6.0 at the rate of 8 ml/hr. Equilibration of the column with the same buffer was done prior to the layering of the substance (50 mg) in this buffer on top of the column; 5 ml fractions were collected. The fractions corresponding to the product peaks were pooled, concentrated, dialyzed and lyophilized as mentioned in section 3.2. The bulk and the residual phosphate was removed by the procedure also outlined in that section.

4.2.2 Preparation of 4-Isothiocyanato-2,6-pyridinedicarboxylic Acid

This compound was prepared from chelidamic acid (4-oxo-1,4-dihydro-2,6-pyridinedicarboxylic acid) by the route outlined below.



Chelidamic acid

4.2.2.1 4-Chloro-2,6-pyridinedicarboxylic Acid

This was prepared by the method of Koenigs *et al.* [1921]. 22 g (0.11 mole) of chelidamic acid (Aldrich Chemical Company Inc.) was dried for 2 hours at 150°C, finely powdered and quickly mixed together with 68 g of phosphorus pentachloride (0.33 mole) and transferred to a flask fitted with a condenser and a calcium chloride guard tube. A violent reaction soon began, the mixture melted and began to boil. After the evolution of HCl had subsided the mixture was boiled for a further one hour. After cooling, the deep brown solution was slowly poured into 200 g of ice. The chloro acid separated out as a

dark brown solid. The mixture was kept in ice for a further 0.5 h and then filtered. The product was washed with ice cold water and then dried in the oven. After recrystallization from hot water the product was obtained as colourless needles, m.p. 220° (lit. [Koenigs *et al.* 1921] m.p. 220°). The substance decomposed at its melting point with charring. Yield 20 g (90%).

4.2.2.2 4-Amino-2,6-pyridinedicarboxylic Acid

The method of Koenigs *et al.* [1924] was employed for the preparation of this compound. 4 g of 4-chloro-2,6-pyridinedicarboxylic acid (0.02 mole) was treated with 20 ml of 30% W/W ammonia of specific gravity 0.88 in a stainless steel bomb of 120 ml capacity. The bomb was tightly closed and heated by placing it in an oven maintained at 150°C for 12 hours. This was then cooled, opened and the unreacted ammonia and water were evaporated by heating on a waterbath. The dried mass was dissolved in water and then treated with dilute hydrochloric acid until acidic when the amino compound separated out. The solid was filtered and a further small amount was also isolated from the filtrate. The compound was recrystallised from hot water and formed rose coloured crystals, m.p. 298° dec. (lit. [Koenigs *et al.* 1924] m.p. 298°). Yield 3.4 g (93%).

Found: C, 45.8%; H, 3.2%; N, 15.3%, O, 35.6%.

Calculated for $C_7H_6N_2O_4$: C, 46.2%; H, 3.3%; N, 15.4%; O, 35.2%.

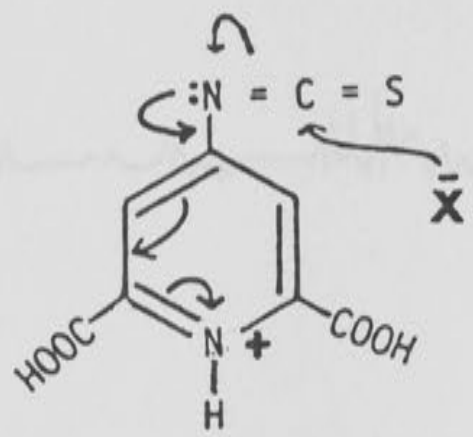
The NMR spectrum of this compound (in ^2H_2O) exhibited a sharp singlet at δ 7.23 due to the aromatic protons, and the mass spectrum showed a molecular ion at m/e 182.

4.2.2.3 4-Isothiocyanato-2,6-pyridinedicarboxylic Acid

The method of Dyson [1961] was employed for the preparation of

this compound. 2 g of 4-amino-2,6-pyridinedicarboxylic acid (0.01 mole) was dissolved in 1 litre of water, the large volume of water being necessary because of the low solubility of this compound. 2.53 g (0.022 mole) of thiophosgene (Fluka) was added to this solution over a period of 30-60 minutes. The solution was then stirred for a further 30 minutes and filtered. The product was washed with cold water and dried *in vacuo*. The crude product was used without further purification, for although the mass spectrum showed a parent peak at m/e 224 the compound decomposed on attempted recrystallisation.

The instability of this isothiocyanate may be the result of rapid nucleophilic attack at this functional group since this would be enhanced by the low electron density of the pyridinium nitrogen atom (4.5).



4.5

where $^{\ominus}X$ is a nucleophile like $^{\ominus}OH$, $-NH_2$ etc.

4.2.3 Reaction of 4-Isothiocyanato-2,6-pyridinedicarboxylic Acid with Ribonuclease A (Preparation of 4-IDPA-RNase A)

200 mg of ribonuclease A (14.5 micro moles) was dissolved in 10 ml of 0.1 M phosphate buffer (pH 7.0). 4-Isothiocyanato-2,6-pyridinedicarboxylic acid (58 micro moles) was added to this solution in two portions at an interval of 12 hours. The solution was stirred

at room temperature for 24 hours and then dialyzed and lyophilized.

The products of this preparation were separated chromatographically as described in section 4.2.1. 4-IDPA was reacted with the peptide Gly-Gly-Gly under analogous conditions.

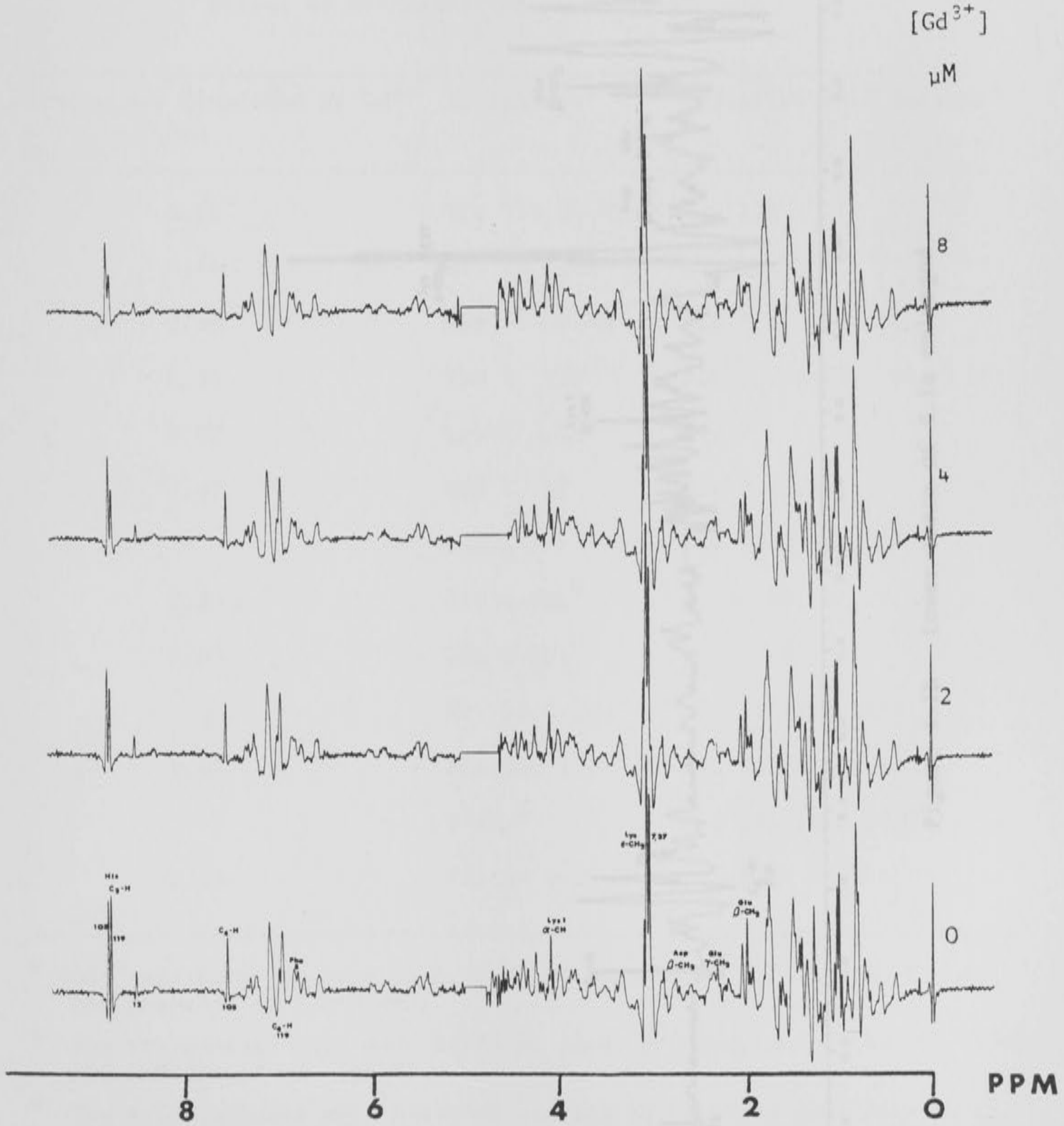


Figure 4.1a The effect of Gd^{3+} addition to ribonuclease A studied at 270 MHz. 5.8 mM of RNase A containing 0.1 M NaCl at pH 5.0 and 21°C was used. Additions were made from a 1 mM stock solution of $Gd(NO_3)_3.6H_2O$.

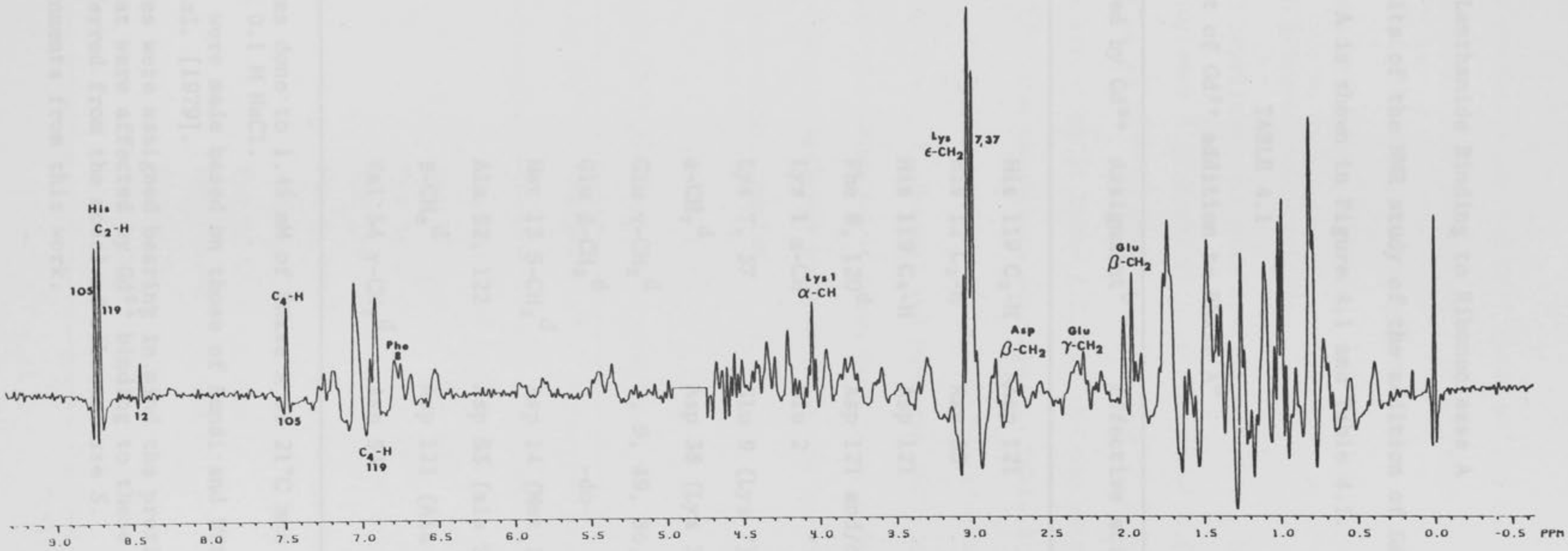


Figure 4.1b Lower trace of 4.1a enlarged.

4.3 RESULTS

4.3.1 Studies of Lanthanide Binding to Ribonuclease A

The results of the NMR study of the addition of Gd^{3+} to 2.9 mM of ribonuclease A is shown in Figure 4.1 and Table 4.1.

TABLE 4.1

Effect of Gd^{3+} addition to RNase A[a]

Resonances broadened by Gd^{3+} ppm	Assignment[b]	Effective acid residue[c]
8.72	His 119 C_2-H	Asp 121
8.44	His 12 C_2-H	Asp 83
6.96	His 119 C_4-H	Asp 121
6.83	Phe 8, 120[d]	Asp 121 and/or Val 124
4.07	Lys 1 α-CH	Glu 2
3.07	Lys 7, 37	Glu 9 (Lys 7),
	ε-CH_2[d]	Asp 38 (Lys 37)
2.30	Glu γ-CH_2[d]	2, 9, 49, 86, 111
2.00	Glu β-CH_2[d]	-do-
	Met 13 S-CH_3[d]	Asp 14 (Met 13)
1.43	Ala 52, 122	Asp 53 (ala 52)
	β-CH_3[d]	Asp 121 (Ala 122)
1.03	Val 54 γ-CH_3[d]	Asp 53

a Gd^{3+} addition was done to 1.45 mM of RNase A at 21°C and pH 5.0 in the presence of 0.1 M NaCl.

b The assignments were made based on those of Bundi and Wüthrich [1978] and Lenstra *et al.* [1979].

c The acid residues were assigned bearing in mind the proximity of the residues that were affected by Gd^{3+} binding to these acid residues as inferred from the model of ribonuclease S.

d Tentative assignments from this work.

At higher concentrations of Gd^{3+} (>100 μM) all the resonances in the NMR spectrum showed some broadening. In addition to the resonances mentioned in Table 4.1, the β-CH_2 of the aspartic acid residues showed broadening, thus indicating that Gd^{3+} binds to the free carboxyl groups of the aspartic and glutamic acid residues.

A similar experiment was also carried out with a higher concentration of ribonuclease A (5.8 mM) with similar results (Table 4.1).

The addition of Yb^{3+} to ribonuclease A was also studied by NMR and the results are shown in Table 4.2. In the concentration range 0-120 μM of added Yb^{3+}, the resonances mentioned in Table 4.2 showed observable changes. No upfield shifts of resonances (expected for a Yb^{3+} complex) were noticed [cf. Inagaki and Miyazawa 1981] and the small amount of observed broadening of resonances could possibly be due to intermediate rates of exchange between the bound and unbound forms of the lanthanide-enzyme complex.

TABLE 4.2

Effect of Yb^{3+} addition to ribonuclease A[a]

Resonances affected by Yb^{3+} ppm	Assignments[b]	Effective acid residue[c]
8.78	His 105 C_2-H	Val 124
8.57	His 12 C_2-H	Asp 83
7.28	Tyr 76 C^{δ}-H	Asp 53
7.19	Tyr 115 C^{δ}-H	Glu 111
6.80	Phe 8, 120[d]	Asp 121 or Val 124 (Phe 8, 120)
6.45-6.55	Tyr 25 C^{ε}-H	Asp 14
4.07	Lys 1 α-CH	Glu 2
2.25	Glu γ-CH_2[d]	
2.1-2.0	Glu β-CH_2[d]	

Continued ...

TABLE 4.2 (Continued)

a Yb^{3+} addition was done to a 2.9 mM soln. of ribonuclease A in ^2H_2O at pH 5.3 containing 0.1 M NaCl and at a temperature of 20°C.

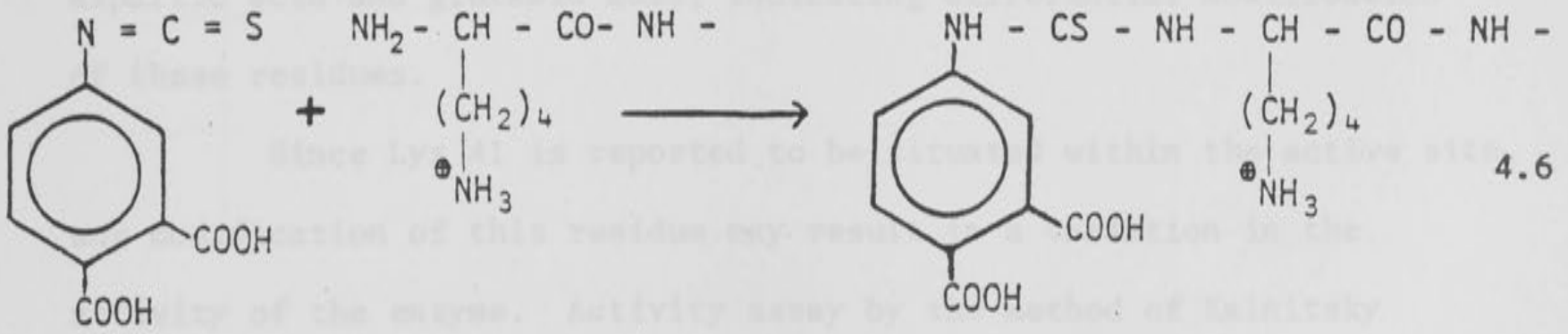
b The assignments were made as previously mentioned in Table 4.1.

c The assignments were made with respect to the acid residues responsible for the effects observed, bearing in mind the factors like proximity and the angular dependence of the pseudo-contact shifts of Yb^{3+}.

d Tentative assignments from this work.

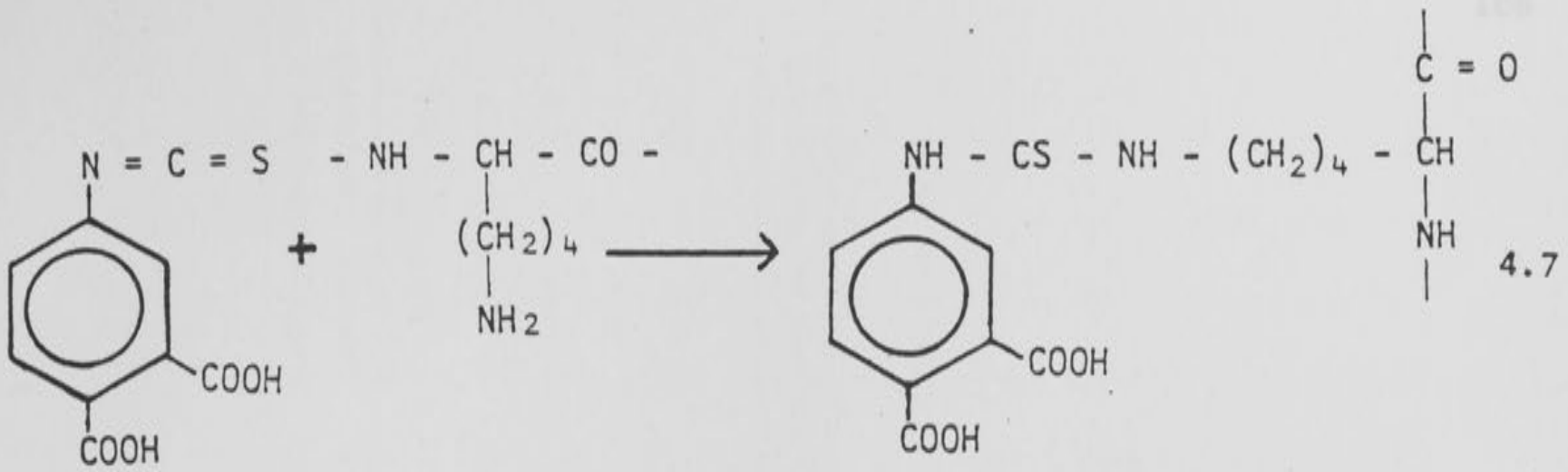
4.3.2 Lanthanide Addition to 4-IPA-RNase A

Ribonuclease A has a lysine residue at the N-terminus, the α-amino group of which exhibits a pK of 7.6 [Bradbury *et al.* 1978]. On the other hand the ε-amino groups of lysine residues exhibit pK values in the range 10.6 - 11.2 [Brown and Bradbury 1975]. By contrast the ε-amino group of Lys 41 has a pK of 8.8 as it is present in a highly positively charged active site [Murdock *et al.* 1966]. Hence at pH 7.2, 4-IPA reacts quite readily with the α-amino group of Lys 1 [Bradbury *et al.* 1978] and the reaction can be depicted as follows



4.6

At this pH, Lys 41 is largely protonated and would only be expected to form a very small amount of the thiocarbamoyl product with 4-IPA.



4.7

The chromatographic separation of the products of the reaction between 4-IPA and ribonuclease A is shown in Figure 4.2. A satisfactory separation was obtained at pH 6.0, with the products eluting much more rapidly than the unreacted ribonuclease A. The net decrease in the positive charge on the protein molecule causes the products to elute at a faster rate on the cation-exchange column. Two major product peaks developed in this chromatogram, a faster moving major product (B) and a slower moving minor product (A).

When amino acid analyses were performed on these two products they established that one lysine residue (in both products) was modified by reaction with 4-IPA (Table 4.3). However considerable differences between the two products were observed in values for the amino acids, aspartic acid and glutamic acid, indicating differential modification of these residues.

Since Lys 41 is reported to be situated within the active site, any modification of this residue may result in a variation in the activity of the enzyme. Activity assay by the method of Kalnitsky *et al.* [1959] was carried out on both these products as well as on ribonuclease A itself and both derivatives were found to exhibit 80% of the activity of the native enzyme.

The progressive addition of Gd^{3+} to both of the products of 4-IPA-RNase A (i.e. products A, B above) was studied by ^{1}H NMR

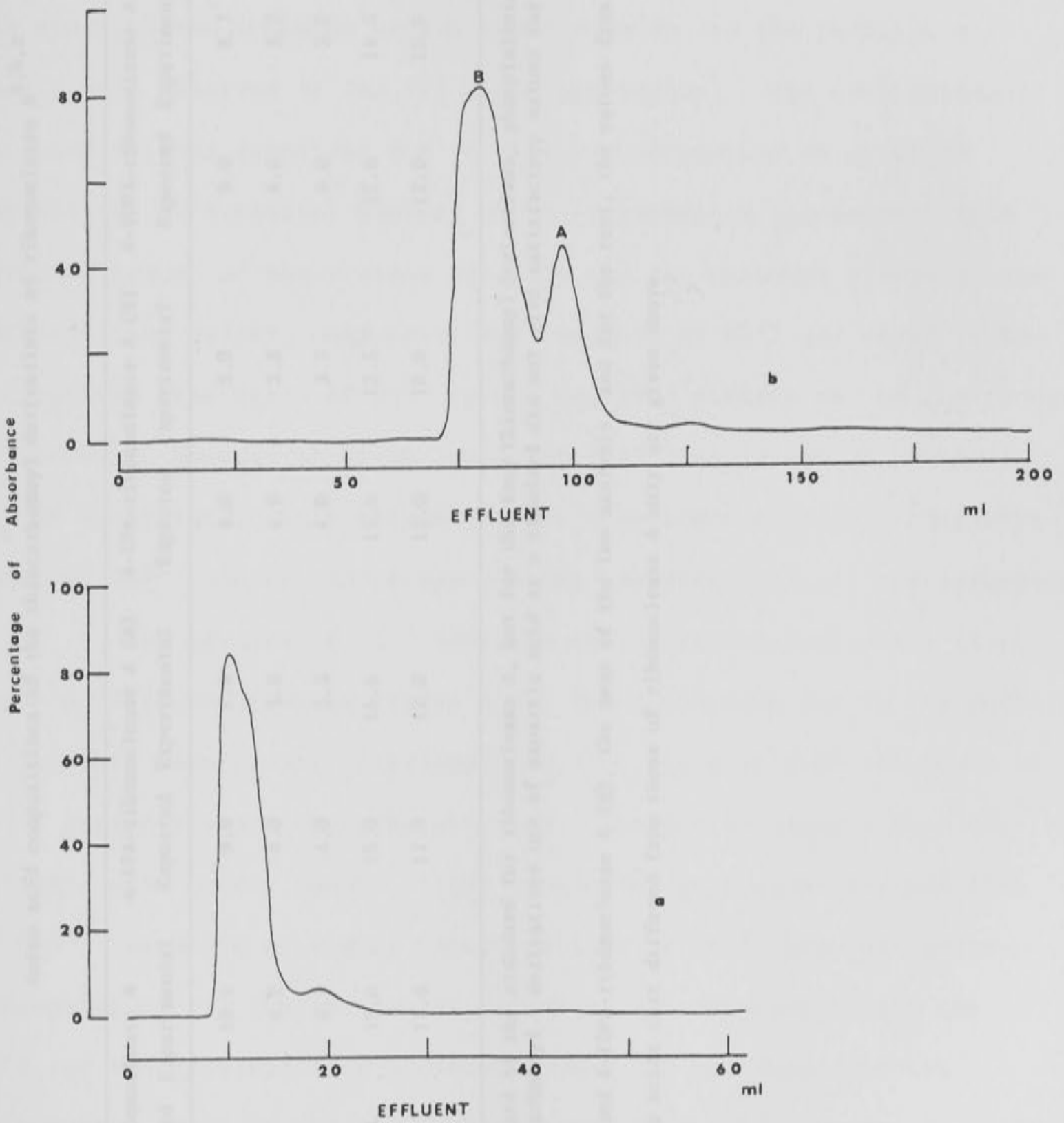


Figure **4.2** Chromatography of 4-IPA-RNase A.
(a) Analytical column. 0.6 x 40 cm column of Bio-Rex 70 (200-400 mesh) ion-exchange resin eluted with 0.2 M PO_4^{3-} buffer, pH 6.0 at a flow rate of 6 ml/hr. Amount laid, 10 mg.
(b) Preparatory column. 1.2 x 100 cm of the column packed with the above resin. Eluent, 0.2 M phosphate buffer at pH 6.0. Eluting rate, 8 ml/hr. Fractions, 5 ml. Amount laid, 50 mg.

TABLE 4.3

Amino acid compositions of the thiocarbamoyl derivatives of ribonuclease A[a,b,c]

Amino acid	Ribonuclease A		4-IPA-ribonuclease A (A)		4-IPA-ribonuclease A (B)		4-IDPA-ribonuclease A (C)		4-IDPA-ribonuclease A (D)	
	Expected	Experimental	Expected	Experimental	Expected	Experimental	Expected	Experimental	Expected	Experimental
Lysine	10.0	10.1	9.0	9.4	8.0	8.9	9.0	8.7	8.0	9.4
Histidine	4.0	4.2	4.0	3.8	4.0	3.8	4.0	3.7	4.0	2.7
Arginine	4.0	4.1	4.0	3.3	4.0	3.7	4.0	3.2	4.0	2.8
Aspartic acid	15.0	15.0	15.0	15.6	15.0	12.5	15.0	14.3	15.0	12.8
Glutamic acid	12.0	11.8	12.0	12.0	12.0	10.0	12.0	10.9	12.0	12.4

a Aspartic acid was used as the standard for ribonuclease A, but for the four thiocarbamoyl derivatives, phenylalanine, was used as the standard. For the four thiocarbamoyl derivatives use of aspartic acid as a standard did not give satisfactory values for most of the amino acids while phenylalanine did.

b For ribonuclease A and 4-IDPA-ribonuclease A (D), the mean of the two analysis, and for the rest, the values from one analysis are given.

c The values for amino acids that differed from those of ribonuclease A only are given above.

spectroscopy. The addition of Gd^{3+} to product A (4-IPA-RNase A (A)) was carried out at pH 5.0 where all the carboxylic acid groups (phthalic acid and the protein) would be more or less in their anionic form. The α-CH resonance of Lys 1 was not observed around 4.1 ppm indicating that the α-amino group of this residue had reacted with the 4-IPA (as expected the aromatic proton resonances due to the phthalic acid moiety were observed in the 7.5 - 8.0 ppm region). The α-CH resonance may have shifted downfield due to this derivatisation. In order to exploit the differential binding of the lanthanides between the free carboxyl groups of the protein molecule and the carboxyl groups of the phthalic acid moiety, only very small amounts of Gd^{3+} was added to the derivatives (the ratio of Gd^{3+} to the modified protein was of the order of $0\text{-}10^{-2}$). However at lower levels of $[Gd^{3+}]$ no selective broadening of the phthalic acid resonances was observed. At higher concentrations of Gd^{3+} (>50 μM) there was general broadening of all the resonances.

The results of Gd^{3+} addition to the second derivative (4-IPA-RNase A (B)) are shown in Figure 4.3. The resonances due to the phthalic acid moiety were clearly distinguished, but again no α-CH resonance of Lys 1 around 4.1 ppm could be observed. The proton signals from the phthalic acid moiety showed slight broadening on progressive addition of Gd^{3+}. As above at higher concentrations of Gd^{3+} there was general broadening of all the resonances. Some of the resonances like the $\gamma\text{-}CH_2$ of the glutamic acid residues around 2.3 ppm showed marked broadening. Most of the other broadenings that were observed in these experiments were similar to those described in section 4.3.1 for ribonuclease A itself. Also there were changes (broadening) observed in the region 3.3 - 4.5 ppm corresponding to the α-CH resonances of the amino acid residues of the protein. Apart from these effects there was

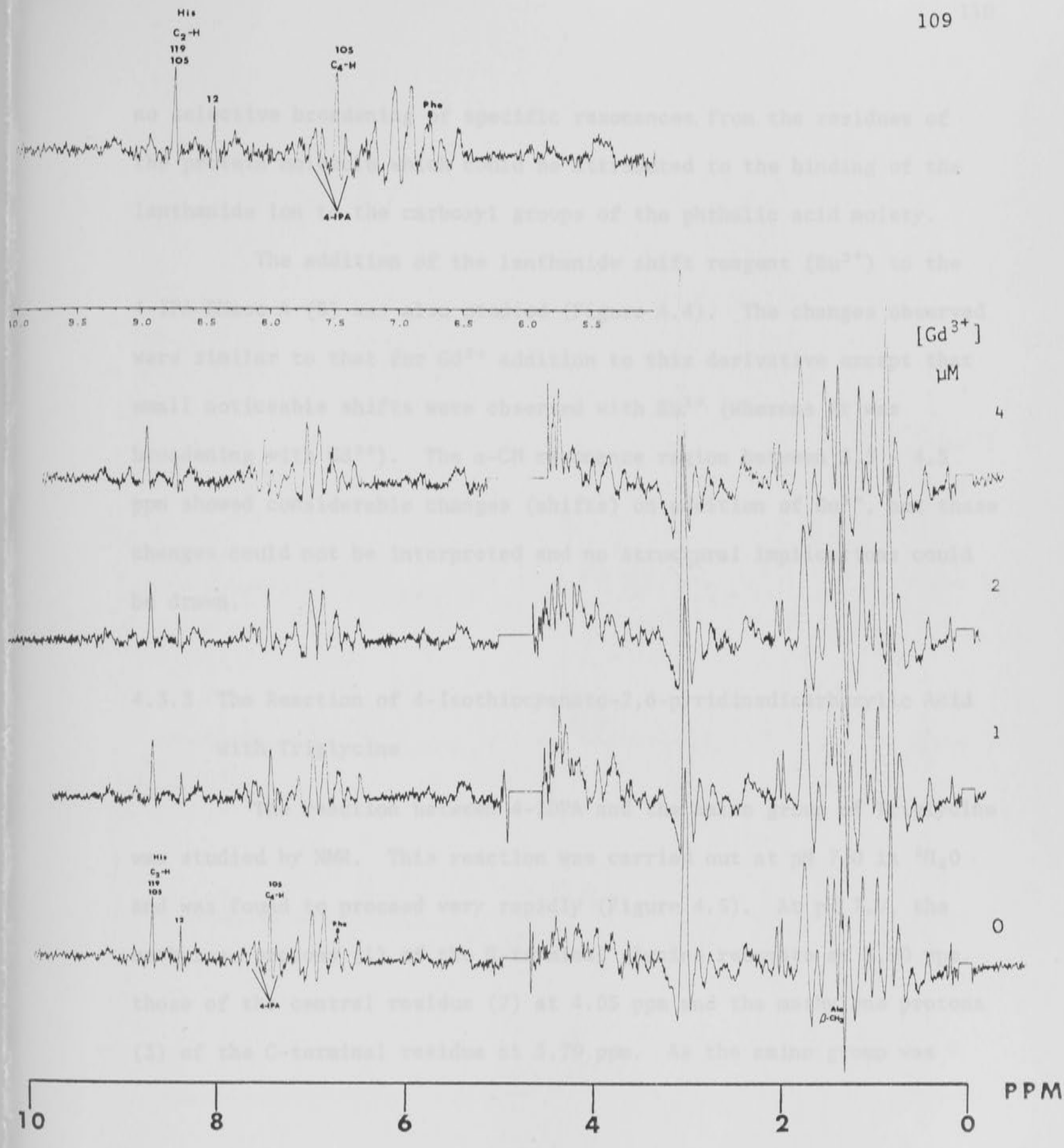


Figure 4.3 The effect of Gd^{3+} addition to 4-IPA-RNase A B) studied with 1.45 mM of the protein solution containing 0.1 M NaCl at pH 5.0 and 21°C.

no selective broadening of specific resonances from the residues of the protein molecule which could be attributed to the binding of the lanthanide ion to the carboxyl groups of the phthalic acid moiety.

The addition of the lanthanide shift reagent (Eu^{3+}) to the 4-IPA-RNase A (B) was also studied (Figure 4.4). The changes observed were similar to that for Gd^{3+} addition to this derivative except that small noticeable shifts were observed with Eu^{3+} (whereas it was broadening with Gd^{3+}). The α-CH resonance region between 3.3 - 4.5 ppm showed considerable changes (shifts) on addition of Eu^{3+}, but these changes could not be interpreted and no structural implications could be drawn.

4.3.3 The Reaction of 4-Isothiocyanato-2,6-pyridinedicarboxylic Acid with Triglycine

The reaction between 4-IDPA and the amino group of Triglycine was studied by NMR. This reaction was carried out at pH 7.0 in ^2H_2O and was found to proceed very rapidly (Figure 4.5). At pH 7.0, the methylene protons (1) of the N-terminal glycine resonate at 3.90 ppm, those of the central residue (2) at 4.05 ppm and the methylene protons (3) of the C-terminal residue at 3.79 ppm. As the amino group was

$$\overset{1}{\overset{\oplus}{N}H_3 - CH_2} - CO - NH - \overset{2}{CH_2} - CO - NH - \overset{3}{CH_2} - COO^{\ominus} \qquad 4.8$$

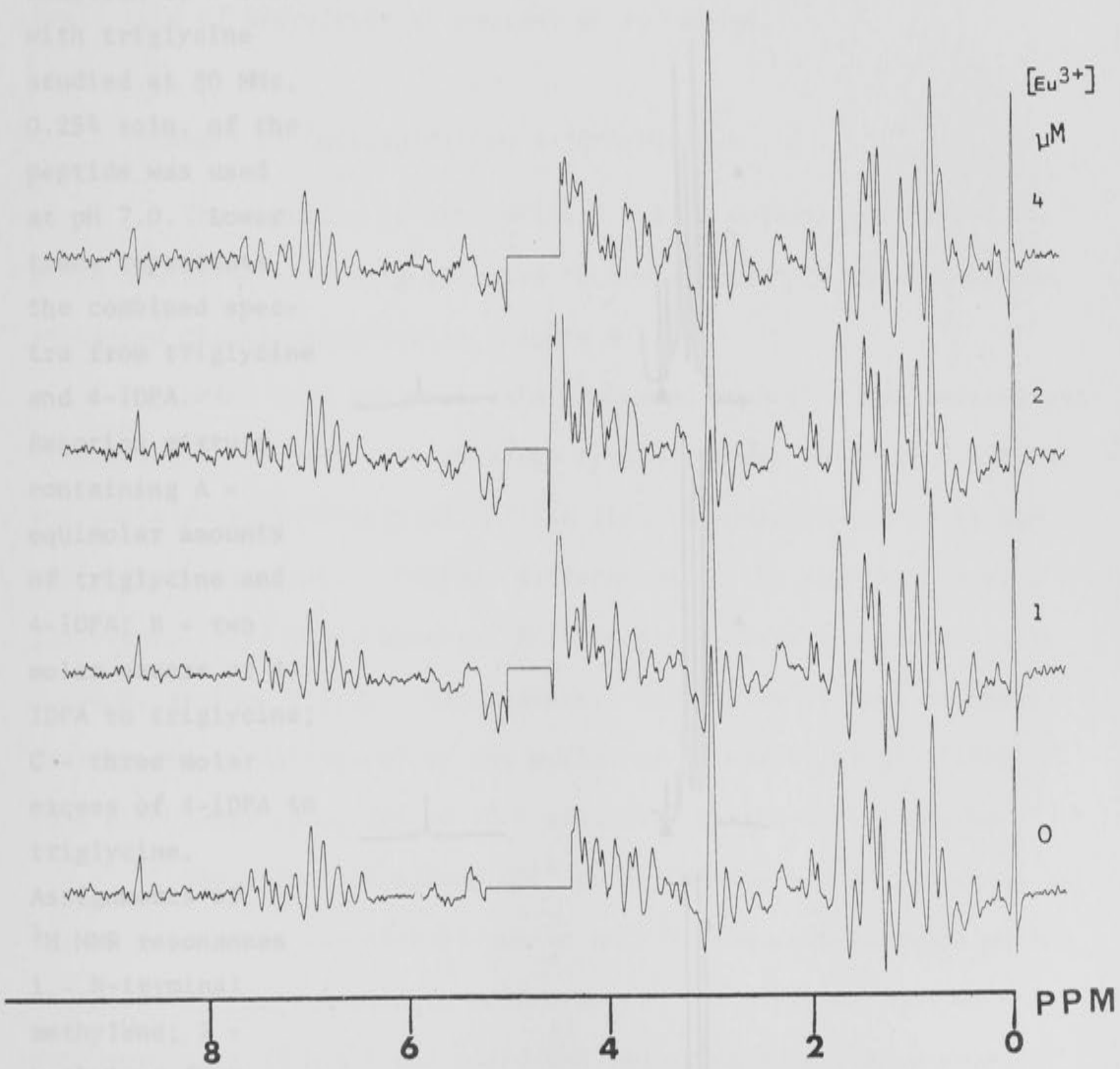


Figure 4.4 The spectra of 4-IPA-RNase A (B) showing the effect of Eu^{3+} addition.

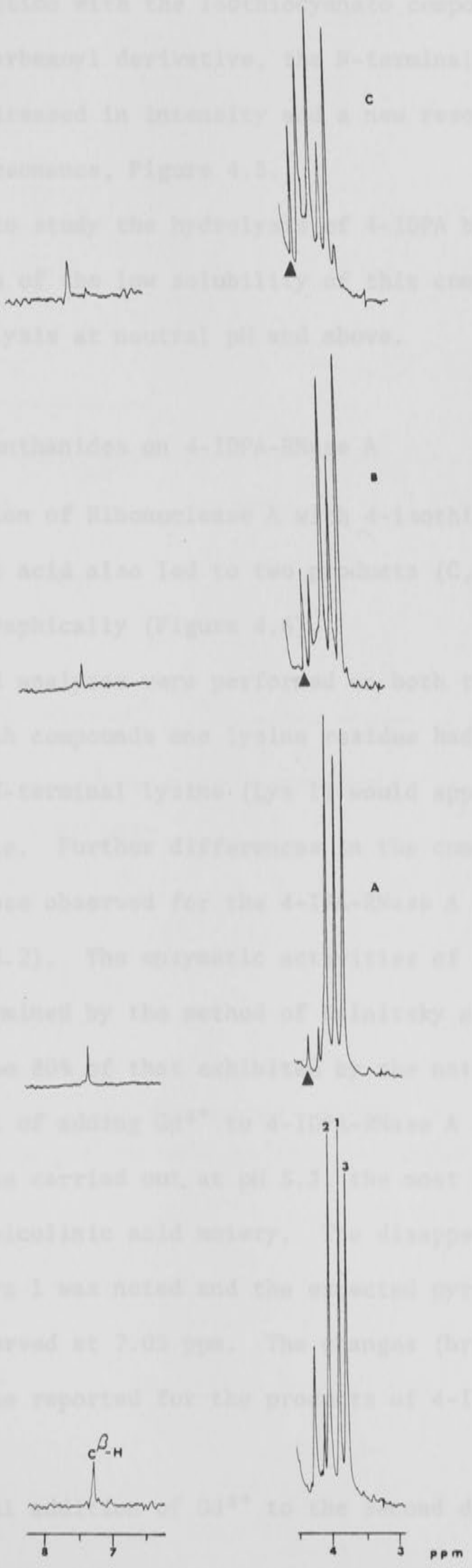


Figure 4.5
Reaction of 4-IDPA with triglycine studied at 80 MHz. 0.25% soln. of the peptide was used at pH 7.0. Lower trace represents the combined spectra from triglycine and 4-IDPA. Reaction mixture containing A - equimolar amounts of triglycine and 4-IDPA; B - two molar excess of 4-IDPA to triglycine; C - three molar excess of 4-IDPA to triglycine. Assignments of ^{1}H NMR resonances 1 - N-terminal methylene; 2 - methylene from second residue; 3 - C-terminal methylene.

modified by its reaction with the isothiocyanato compound to form the corresponding thiocarbamoyl derivative, the N-terminal methylene proton resonance decreased in intensity and a new resonance appeared close to the H^2HO resonance, Figure 4.5.

Attempts to study the hydrolysis of 4-IDPA by NMR were unsuccessful because of the low solubility of this compound and the rapid rate of hydrolysis at neutral pH and above.

4.3.4 Effects of Lanthanides on 4-IDPA-RNase A

The reaction of Ribonuclease A with 4-isothiocyanato-2,6-pyridinedicarboxylic acid also led to two products (C, D) which were separated chromatographically (Figure 4.6).

Amino acid analyses were performed on both these derivatives (Table 4.3). In both compounds one lysine residue had been modified, and the accessible N-terminal lysine (Lys 1) would appear to be the most likely candidate. Further differences in the composition of C and D paralleled those observed for the 4-IPA-RNase A products (i.e. A and B, section 4.3.2). The enzymatic activities of both of these derivatives as determined by the method of Kalnitsky *et al.* [1959] were also found to be 80% of that exhibited by the native enzyme.

The effect of adding Gd^{3+} to 4-IDPA-RNase A (C) is shown in Figure 4.7. This was carried out at pH 5.3, the most suitable pH for chelation by the dipicolinic acid moiety. The disappearance of the α-CH resonance of Lys 1 was noted and the expected pyridine-aromatic resonances were observed at 7.05 ppm. The changes (broadening) observed were similar to those reported for the products of 4-IPA-RNase A (section 4.3.2).

The gradual addition of Gd^{3+} to the second derivative

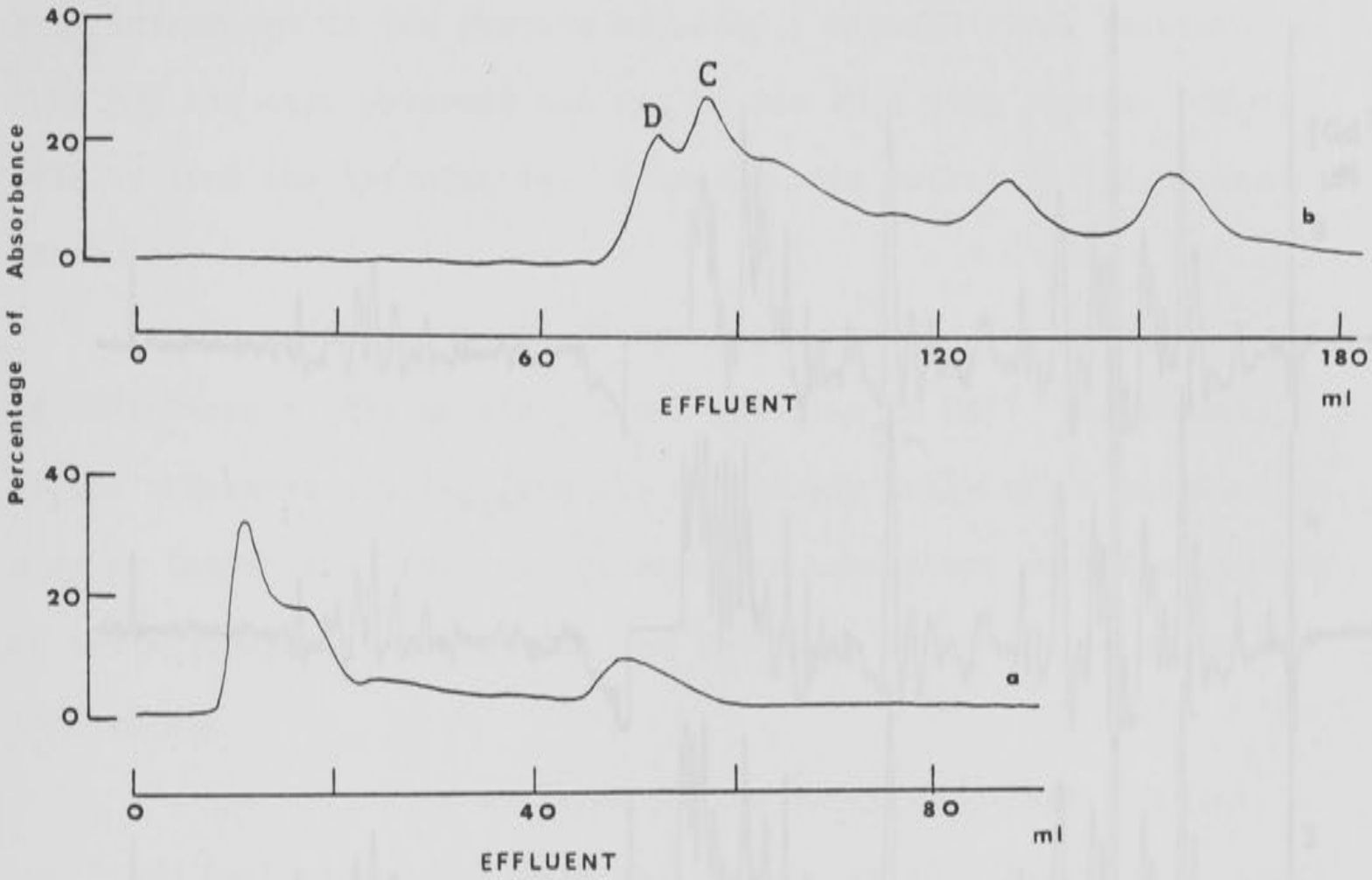


Figure 4.6 Chromatography of 4-IDPA-RNase A
(a) Analytical column. (b) Preparatory column.
The other conditions are the same as that described in Figure 4.2.

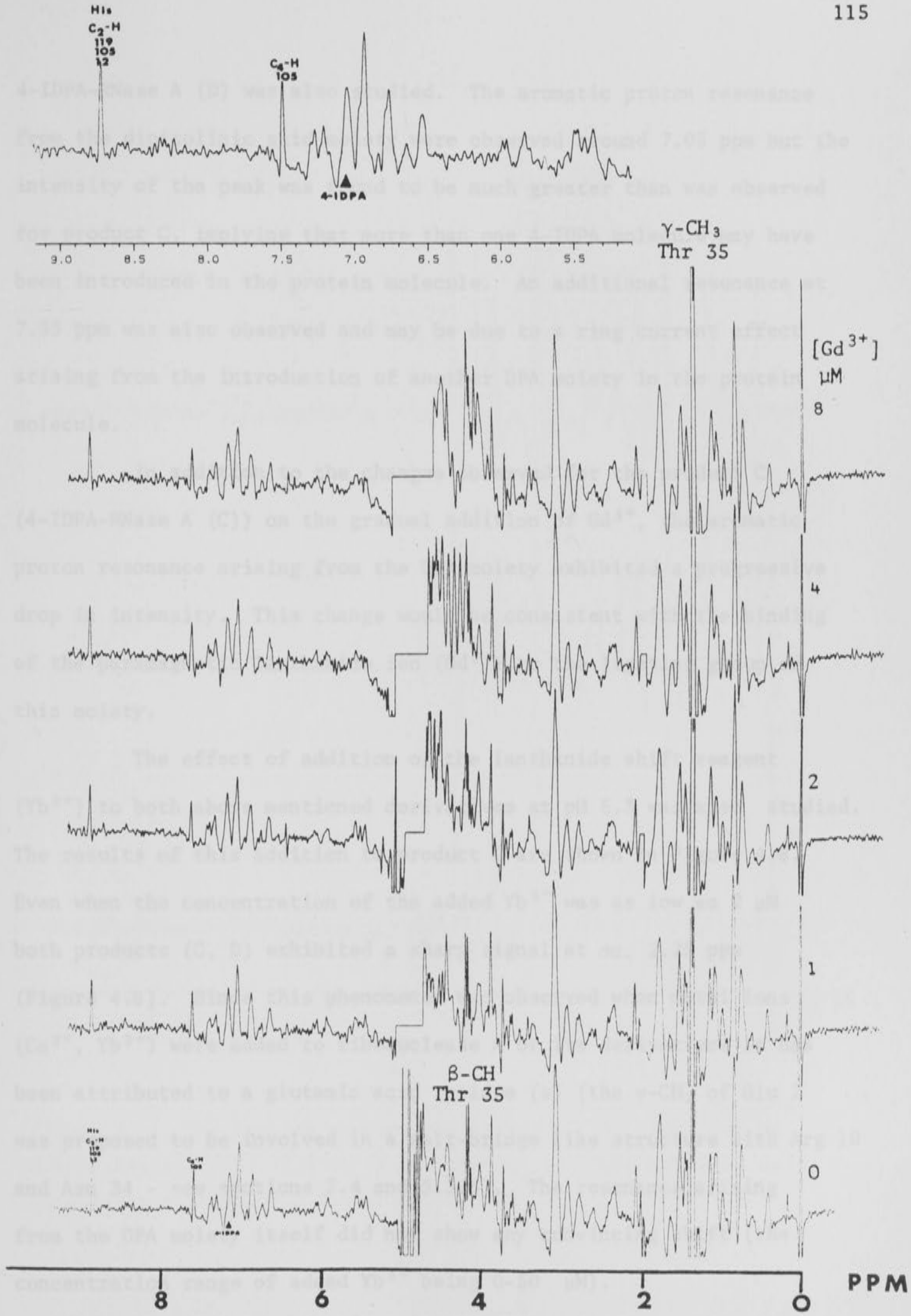


Figure 4.7 The spectra showing the effect of Gd^{3+} addition to 4-IDPA-RNase A (C). Protein = 1.45 mM; NaCl = 0.3 M; pH = 5.3; Temperature = 20°C.

4-IDPA-RNase A (D) was also studied. The aromatic proton resonance from the dipicolinic acid moiety were observed around 7.05 ppm but the intensity of the peak was found to be much greater than was observed for product C, implying that more than one 4-IDPA molecule may have been introduced in the protein molecule. An additional resonance at 7.55 ppm was also observed and may be due to a ring current effect arising from the introduction of another DPA moiety in the protein molecule.

In addition to the changes observed for the product C (4-IDPA-RNase A (C)) on the gradual addition of Gd^{3+}, the aromatic proton resonance arising from the DPA moiety exhibited a progressive drop in intensity. This change would be consistent with the binding of the paramagnetic lanthanide ion (Gd^{3+}) to the ligating group of this moiety.

The effect of addition of the lanthanide shift reagent (Yb^{3+}) to both above mentioned derivatives at pH 5.3 was also studied. The results of this addition to product C are shown in Figure 4.8. Even when the concentration of the added Yb^{3+} was as low as 2 μM both products (C, D) exhibited a sharp signal at *ca.* 2.25 ppm (Figure 4.8). Since this phenomenon was observed when metal ions (Ca^{2+}, Yb^{3+}) were added to ribonuclease A or its derivatives it has been attributed to a glutamic acid residue (s) (the γ-CH_2 of Glu 2 was proposed to be involved in a salt-bridge like structure with Arg 10 and Asn 34 - see sections 3.4 and 5.3.6). The resonance arising from the DPA moiety itself did not show any convincing shift (the concentration range of added Yb^{3+} being 0-50 μM).

The addition of Yb^{3+} to 4-IDPA-RNase A (D) was also studied, the effects observed being the same as mentioned above for product C.

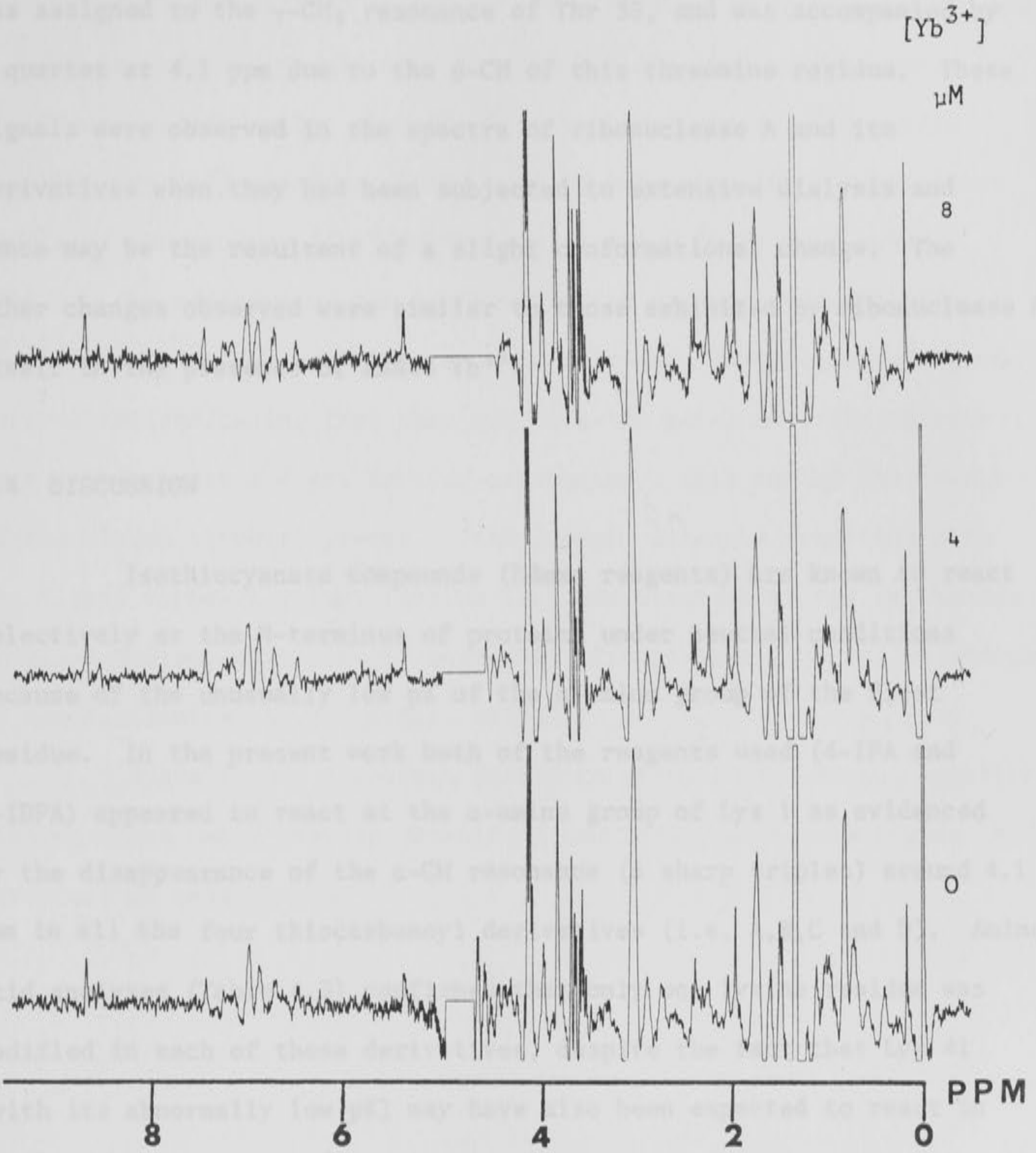


Figure 4.8 The spectra showing the effect of Yb^{3+} addition on 4-IDPA-RNase A (C). 2.9 mM of the protein solution was used under conditions mentioned in Figure 4.7 .

An intense doublet at 1.3 ppm (in the spectra of products C and D) was assigned to the γ-CH_3 resonance of Thr 35, and was accompanied by a quartet at 4.1 ppm due to the β-CH of this threonine residue. These signals were observed in the spectra of ribonuclease A and its derivatives when they had been subjected to extensive dialysis and hence may be the resultant of a slight conformational change. The other changes observed were similar to those exhibited by ribonuclease A itself in the presence of added Yb^{3+}.

4.4 DISCUSSION

Isothiocyanato compounds (Edman reagents) are known to react selectively at the N-terminus of proteins under neutral conditions because of the unusually low pK of the α-amino group of the first residue. In the present work both of the reagents used (4-IPA and 4-IDPA) appeared to react at the α-amino group of Lys 1 as evidenced by the disappearance of the α-CH resonance (a sharp triplet) around 4.1 ppm in all the four thiocarbamoyl derivatives (i.e. A,B,C and D). Amino acid analyses (Table 4.3) confirmed that only one lysine residue was modified in each of these derivatives, despite the fact that Lys 41 (with its abnormally low pK) may have also been expected to react in the presence of excess reagent.

The enzymatic activity measurements showed that all four derivatives exhibited similar activity (*ca.* 80% of native enzyme), so that the introduction of one or more ligand groups into the protein molecule does not reduce the activity appreciably. The derivatives B and D certainly have residues other than Lys 1 modified by reaction with 4-IPA and 4-IDPA (as indicated by the chromatographic behaviour and amino acid analyses) but the site (s) of reaction could not be identified with certainty.

The results of the addition of lanthanides to the four thiocarbamoyl derivatives (A,B,C,D) are described in sections 4.3.2 and 4.3.4. In order to ensure that the lanthanides bind predominantly to the ligand moiety rather than the free carboxyl groups of aspartic and glutamic acid residues of the protein molecule, low concentration levels of lanthanides ($[Ln^{3+}]/[Protein] = 0\text{-}3 \times 10^{-2}$) were added. Although the addition of the lanthanides did produce specific effects on the resonances of both the phthalic acid and DPA moieties of these derivatives indicating that they did bind the metal ion, the effects were rather minor and not totally convincing. This may be the result of the eleven carboxyl groups of the protein molecule competing with the ligand carboxyl groups for the low concentration of the lanthanide metal ion present and in fact comparable effects were observed on addition of the lanthanides to the native enzyme.

Overall this technique failed to produce significant specific changes which could provide useful and convincing data relating to the structure of this enzyme.

CHAPTER 5

NMR STUDIES ON THE INTERACTIONS OF SOME SPORE CONSTITUENTS

5.1 INTRODUCTION

A typical bacterial spore is shown in Figure 5.1 [Gould and Hurst 1969]. The outer exosporium has a very complex structure with a phospholipoprotein composition similar to those of cell membranes along with some sugars (10.4%), glucosamine (11.2%) and a non-hydrolysable fraction consisting mostly of degraded carbohydrate [Murrell 1969]. Following the exosporium is the coat which forms the major part of the spore occupying 50% of the spore volume and is made up of several layers. These layers are composed of chemically very stable disulphide-rich proteins (with structures similar to that of keratin),1-2% lipid, phosphorus and inorganic matter and sometimes glycopeptide or hexosamine. Following the coat is a membrane like structure designated as the innermost or inner coat which separates the coat and the next constituent of spores, the cortex. The cortex consists largely of a muropeptide polymer (murein) which resembles those found in vegetative cell walls. The muropeptide polymers are cross linked sugar-proteins, having molecular weights of the order of 10000 Daltons and are made up of amino sugars, amino acids and small amounts of ash (P and mainly Ca). In addition they contain muramic lactam and L-alanine substituted muramic acids. The germ cell wall is the layer following the cortex and is composed

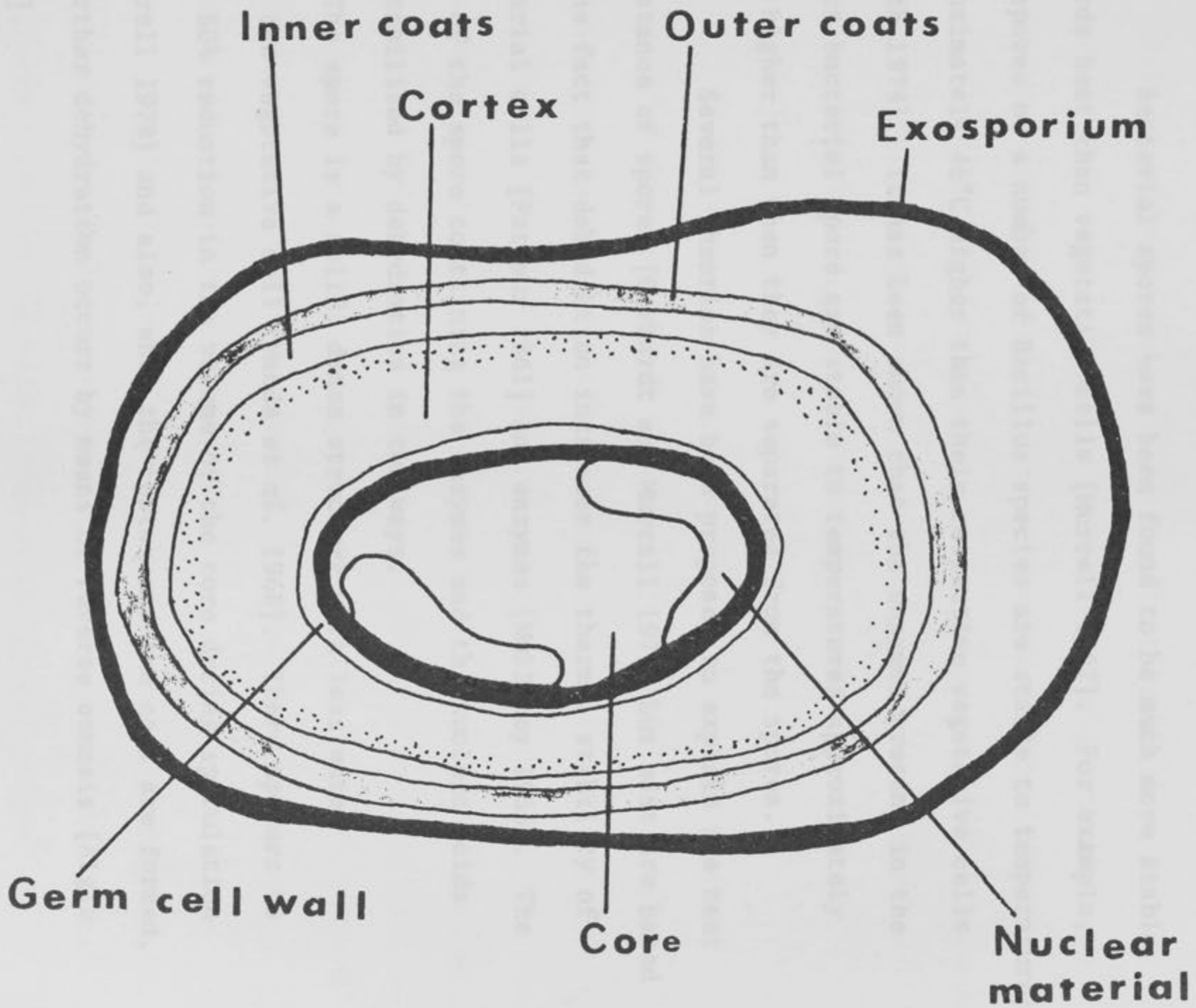


Figure 5.1 A typical bacterial spore [Gould and Hurst 1969].

of a diaminepimelate-containing muropeptide. The core forms the innermost part of the spore and contains enzymes, ribosomes, RNA, DNA, sugars, Ca^{2+}, dipicolinic acid (DPA), free amino acids, 3-phosphoglyceric acid (3-PGA), sulpholactic acid, cytochrome and heamatin.

Bacterial spores have been found to be much more stable towards heat than vegetative cells [Murrell 1967]. For example, the spores of a number of Bacillus species are stable to temperatures approximately 46°C higher than their respective vegetative cells [Warth 1978]. It has been shown that the enzymes present in the intact bacterial spore are stable to temperatures approximately 38°C higher than when they are separated from the spores.

Several theories have been proposed to explain the heat resistance of spores [Gerhardt and Murrell 1978] but most are based on the fact that dehydration increases the thermal stability of bacterial cells [Pasteur 1861] and enzymes [Mullaney 1966]. The core of the spore containing the enzymes and the nucleic acids is stabilised by dehydration in two ways.

1. The spore is a solid, dense structure with less water than the vegetative cell [Maeda *et al.* 1968]. There appears to be a 50% reduction in the volume of the core during sporulation [Murrell 1978] and also, when the cortex and the coat are formed, a further dehydration occurs by means of reverse osmosis [Algie 1980].
2. The core is also considered to be less hydrated than the outer constituents namely the cortex and the coat of the spore. This preferential dehydration may occur in various ways. It may

be due to contraction of the cortex which can exert pressure on the core [Lewis *et al.* 1960], by anisotropic swelling of the cortex [Alderton and Snell 1963; Warth 1977], by cortex expansion due to electrostatic repulsion of the negatively charged carboxyl groups of the muropeptide polymer against the heavily cross-linked protein coat which may compress the core [Gould and Dring 1975] or by the development of an osmotic pressure between the cortex and the core that may cause dehydration of the core [Gould and Dring 1975]. In the presence of an electrolyte such osmotic pressure will increase but electrostatic repulsion between carboxyl groups will decrease and hence these two effects counteract one another [Baillie and Murrel 1974].

^{1}H NMR studies have previously been undertaken to test the second of these postulates [Bradbury *et al.* 1981] by studying the transverse relaxation rate ($1/T_2$) of the spores, the coat and (coat + cortex) preparations for five species of spores. It was found that water in the core was more mobile than that in the outer integuments. The results of this study, together with sorption studies on spores and spore components [Watt 1981], show that there is no evidence to support the concept of dehydration of the core as compared with the cortex and coat.

Besides the dehydration core theory, stabilisation of spore components has been attributed to cross-linking between cell biopolymers [Black and Gerhardt 1962] or to "calcification" of the interior of the spores involving chelation of divalent metal ions by spore ligands [Powell 1953; Rieman 1963; Halvorson 1953; Tang *et al.* 1968]. It has also been suggested that the high content of coordination complexes may be responsible for the hydrophobic nature of the core and hence the above mentioned theories may not

be contradictory [Grecz and Smith 1966].

Bacterial spores contain large amounts of divant metal ions such as Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} [Murrell 1969]. Among these, calcium has been found to be most closely related to the heat resistance. Replacement of calcium with other divalent ions results in a decrease of heat resistance [Murrell 1961] and furthermore, the calcium content of spores has been shown to be approximately 10 times that of vegetative cells.

While the amount of Ca^{2+} in the bacterial endospores is about 1-3% dry weight (which is very much in excess of the other above mentioned divalent metal ions), DPA is present to the extent of 5-15% always in a 1:1 mole ratio with the former [Murrell 1969]. It has been shown that the heat resistance develops sometime after the appearance of DPA [Halvorson 1957; Hashimoto *et al.* 1960] and in some cases is lost before DPA is released [Rode and Foster 1960]. Hence DPA is known to play a prominent role in some other process or processes which directly influence heat resistance [Halvorson and Howitt 1961]. In fact DPA itself has been reported to effect the heat stabilisation of spore enzymes like DPNH oxidase [Doi and Halvorson 1961] and glucose dehydrogenase [Hachisuka *et al.* 1967] in solution.

DPA is a strong chelating agent [Sillén and Martell 1964] and Young [1959] has observed that DPA can form a calcium-DPA-amino acid chelate complex with a variety of amino acids. Similar complexes may exist between calcium-DPA and the protein of the spore and in fact mixed chelates of Ca^{2+}-DPA (Ca-DPA) and some amino acids present in bacterial spores have also been reported [Tang *et al.* 1968]. Thus the combination of DPA, amino acids and cations such as Ca^{2+} through chelation may form a protective cement of low polarity,

low hygroscopicity and generally low chemical reactivity which may protect the vital spore biopolymers against thermal denaturation by a "masking" or "caging" effect [Grecz and Smith 1966].

Heating experiments carried out with enzymes (like ribonuclease A and lactic dehydrogenase) in the presence of Ca-DPA in the dry state showed a positive indication of heat stabilisation of these enzymes by Ca-DPA [Appendix I and Bradbury *et al*. unpublished results]. Also it was shown that the melting temperature of DNA in solution was raised in the presence of Ca-DPA [Murrell 1981].

In the present work an attempt to study the mechanism of the interaction between Ca-DPA and amino acids on the one hand and between Ca-DPA and the proteins on the other, has been made using NMR as the probe. It was hoped that the investigation of these phenomena might lead to an understanding of their involvement in the heat stabilisation of bacterial spores.

5.2 Preparation of Calcium dipicolinate

Five grams of dipicolinic acid was dissolved with warming in one litre of water. This solution (0.03 M) was then slowly added with stirring to a solution (0.03 M) of 4.8 g of calcium acetate (BDH) in a small volume of water. After the addition, the solution was evaporated to three-fourths of the original volume when Ca-DPA separated out as a white powder. The solid was filtered, washed repeatedly with warm as well as cold water and dried. Elemental analysis of this compound showed that a sesquihydrate with 98% purity was obtained.

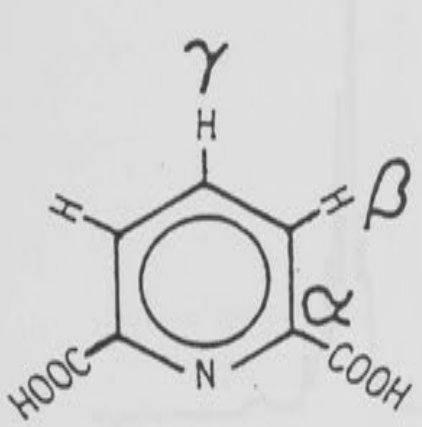
Found: C, 36.5%; H, 2.6%; N, 5.8%; O, 37.9%; Ca, 17.1%.

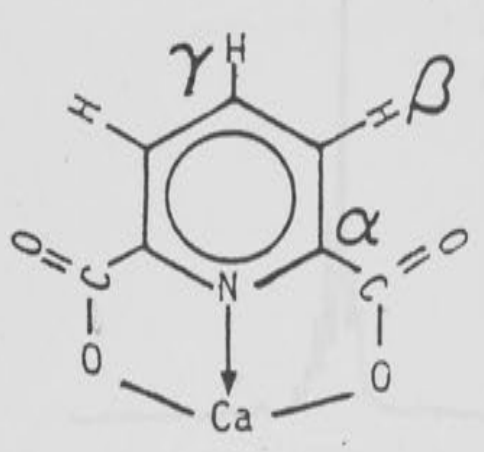
Calculated for $C_7H_3NO_4Ca$: C, 36.2%; H, 2.6%; N, 6.2%; O, 37.7%; Ca, 17.2%.

5.3 RESULTS

5.3.1 ^{1}H NMR Studies of Dipicolinic Acid and Its Calcium Salt

The NMR spectrum of Ca-DPA obtained at pH 7.0 is shown in Figure 5.3. As the three aromatic protons of the dipicolinic acid moiety appear as a sharp singlet at 8.14 ppm, the ortho (C^{β}-H and C^{γ}-H) and the meta (C^{β}-H) couplings (8-10 Hz and 3-4 Hz respectively) are not observed. The NMR spectrum of dipicolinic acid was also recorded at various pH values (Figure 5.2). Below pH 1.0 and above pH 5.5 a sharp singlet was observed for the aromatic protons but between pH 1.0 and 5.5 both Ca-DPA and DPA exhibit spectra with splitting in the broad aromatic resonance envelope. Since the two carboxyl groups of DPA have pKs of 2.16 and 4.76 [Tichane and





5.1

Bennett 1957], at pH values corresponding to the fully protonated and the unprotonated forms, the electronic effects of the two carboxyl groups and the nitrogen of DPA may operate in the same direction so that the three aromatic protons experience the same amount of shielding and deshielding. The splittings observed at intermediate pH values may be due to the difference in the electronic effects experienced by these protons due to the partial dissociation of the carboxyl groups. During the pH titration of DPA from the protonated to the dianionic form, the aromatic proton resonances shift from

Figure 5.2 ^{1}H NMR spectra obtained at 80 MHz of dipicolinic acid as a function of pH.

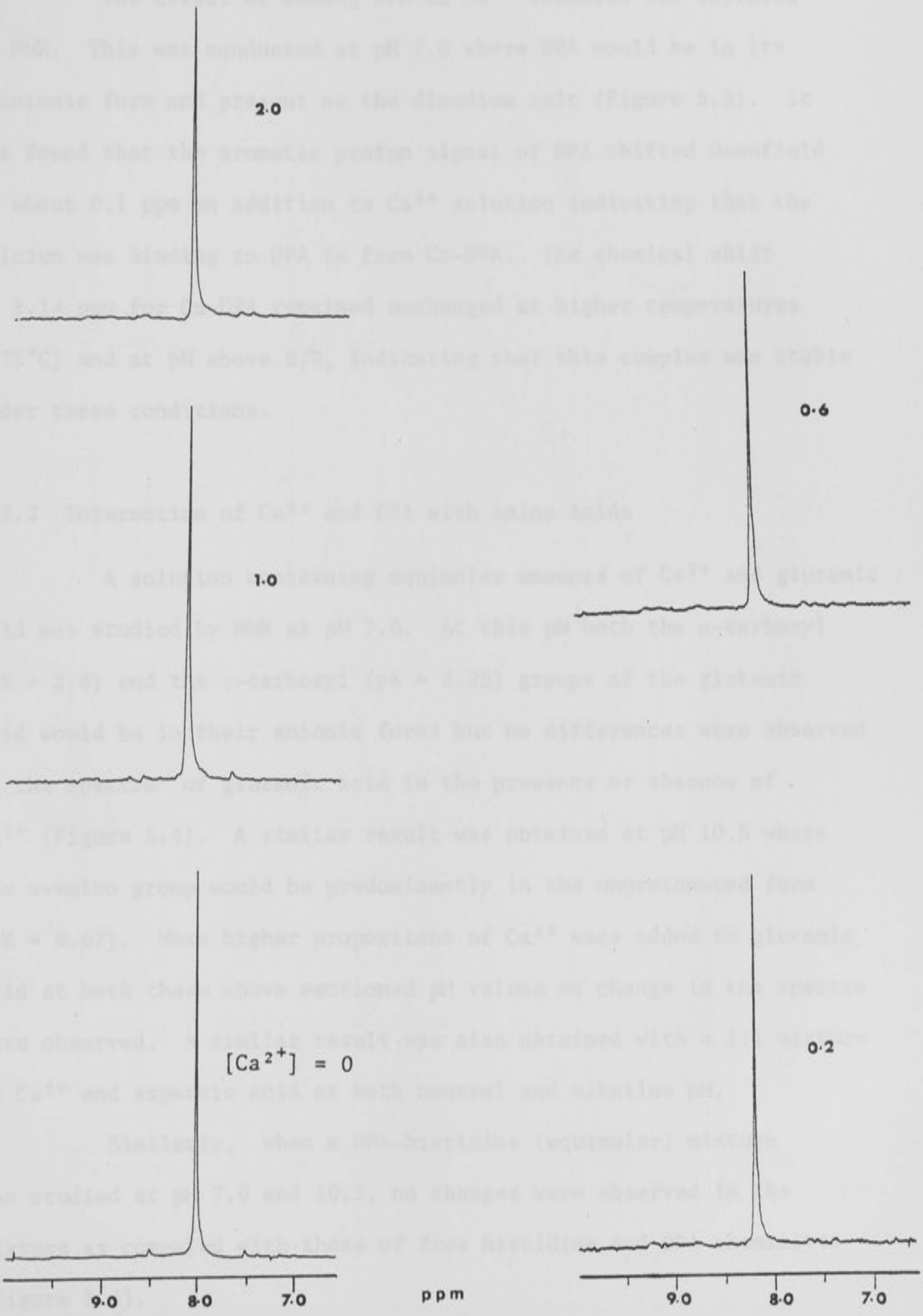


Figure 5.3 ^{1}H NMR spectra of the C^{β} and the C^{γ} protons of DPA obtained at various [DPA]/[Ca^{2+}] ratio at 80 MHz. Ca^{2+} = 2.4 x 10^{-2} M; KCl = 0.1 M; pH = 7.0; Temperature = 30°C.

8.57 ppm to 8.04 ppm (Figure 5.2).

The effect of adding DPA to Ca^{2+} solution was followed by NMR. This was conducted at pH 7.0 where DPA would be in its dianionic form and present as the disodium salt (Figure 5.3). It was found that the aromatic proton signal of DPA shifted downfield by about 0.1 ppm on addition to Ca^{2+} solution indicating that the calcium was binding to DPA to form Ca-DPA. The chemical shift of 8.14 ppm for Ca-DPA remained unchanged at higher temperatures (>75°C) and at pH above 6.0, indicating that this complex was stable under these conditions.

5.3.2 Interaction of Ca^{2+} and DPA with Amino Acids

A solution containing equimolar amounts of Ca^{2+} and glutamic acid was studied by NMR at pH 7.0. At this pH both the α-carboxyl (pK = 2.8) and the γ-carboxyl (pK = 4.25) groups of the glutamic acid would be in their anionic forms but no differences were observed in the spectra* of glutamic acid in the presence or absence of Ca^{2+} (Figure 5.4). A similar result was obtained at pH 10.5 where the α-amino group would be predominantly in the unprotonated form (pK = 9.67). When higher proportions of Ca^{2+} were added to glutamic acid at both these above mentioned pH values no change in the spectra were observed. A similar result was also obtained with a 1:1 mixture of Ca^{2+} and aspartic acid at both neutral and alkaline pH.

Similarly, when a DPA-histidine (equimolar) mixture was studied at pH 7.0 and 10.5, no changes were observed in the mixture as compared with those of free histidine and DPA themselves (Figure 5.4).

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* The assignments of the ^1H resonances of the amino acids are based upon those of Bundi and Wüthrich [1979].

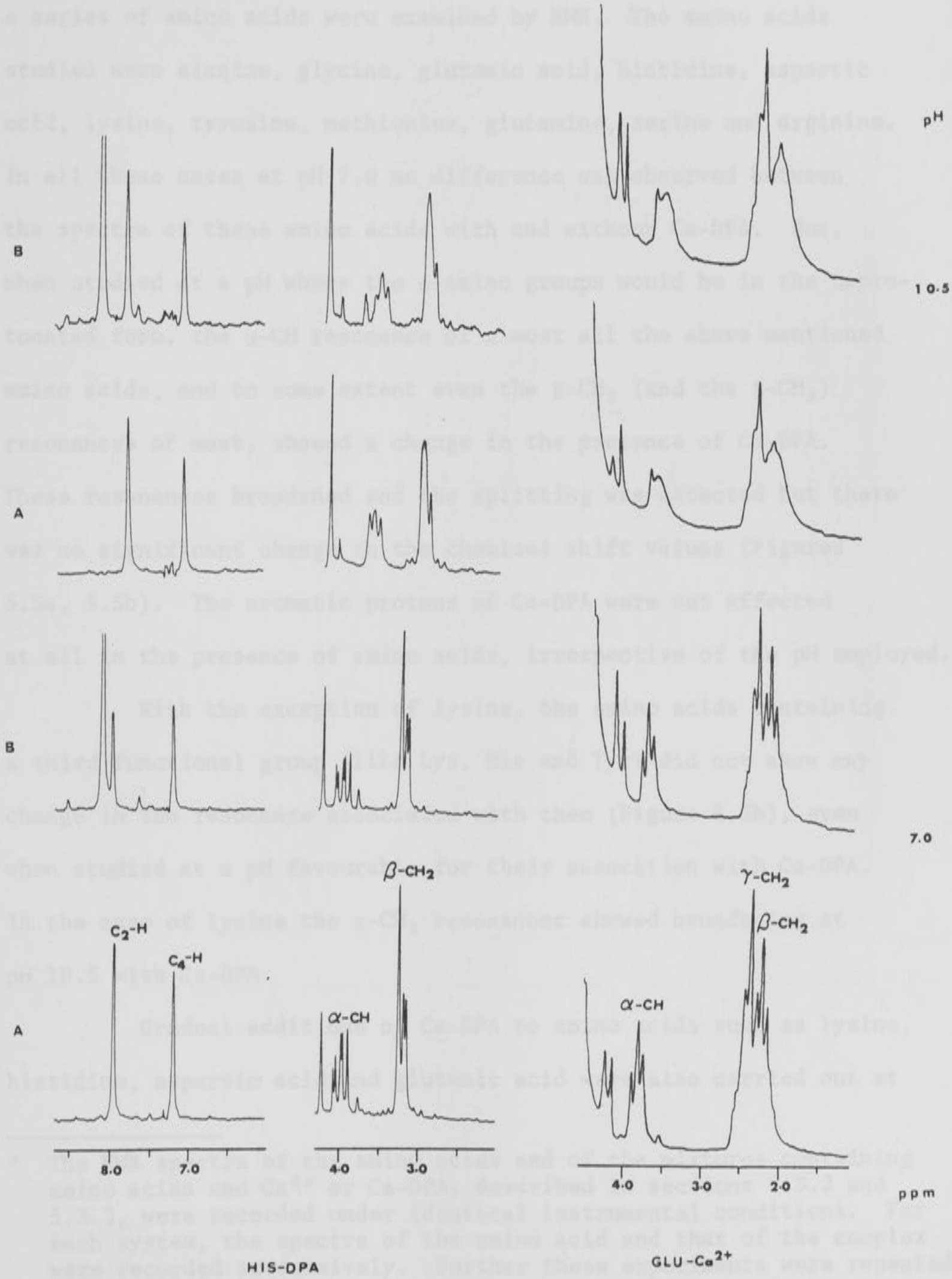

Figure 5.4 ^{1}H NMR spectra of equimolar amounts of His-DPA and Glu-Ca^{2+} mixture obtained at 80 MHz. Solutions were DPA = 2.4 x 10^{-2} M; Glu = 0.062 M; KCl = 0.1 M. Temp = 30°C. Traces A represent the spectra of free amino acids. Traces B those with Ca^{2+} in the case of Glu and DPA in the case of His.

5.3.3 NMR Studies* of Ca-DPA-Amino Acid Systems

Solutions containing equimolar amounts of Ca-DPA and a series of amino acids were examined by NMR. The amino acids studied were alanine, glycine, glutamic acid, histidine, aspartic acid, lysine, tyrosine, methionine, glutamine, serine and arginine. In all these cases at pH 7.0 no difference was observed between the spectra of these amino acids with and without Ca-DPA. But, when studied at a pH where the α-amino groups would be in the unprotonated form, the α-CH resonance of almost all the above mentioned amino acids, and to some extent even the β-CH_2 (and the β-CH_3) resonances of most, showed a change in the presence of Ca-DPA. These resonances broadened and the splitting was affected but there was no significant change in the chemical shift values (Figures 5.5a, 5.5b). The aromatic protons of Ca-DPA were not affected at all in the presence of amino acids, irrespective of the pH employed.

With the exception of lysine, the amino acids containing a third functional group (like Lys, His and Tyr) did not show any change in the resonance associated with them (Figure 5.5b), even when studied at a pH favourable for their assocation with Ca-DPA. In the case of lysine the ε-CH_2 resonances showed broadening at pH 10.5 with Ca-DPA.

Gradual additions of Ca-DPA to amino acids such as lysine, histidine, aspartic acid and glutamic acid were also carried out at

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* The NMR spectra of the amino acids and of the mixtures containing amino acids and Ca^{2+} or Ca-DPA, described in sections 5.3.2 and 5.3.3, were recorded under identical instrumental conditions. For each system, the spectra of the amino acid and that of the complex were recorded successively. Further these experiments were repeated several times to ensure that the observed broadening of the signals from the amino acids in the presence of Ca-DPA, were genuine and did not arise from variations in instrumental conditions.

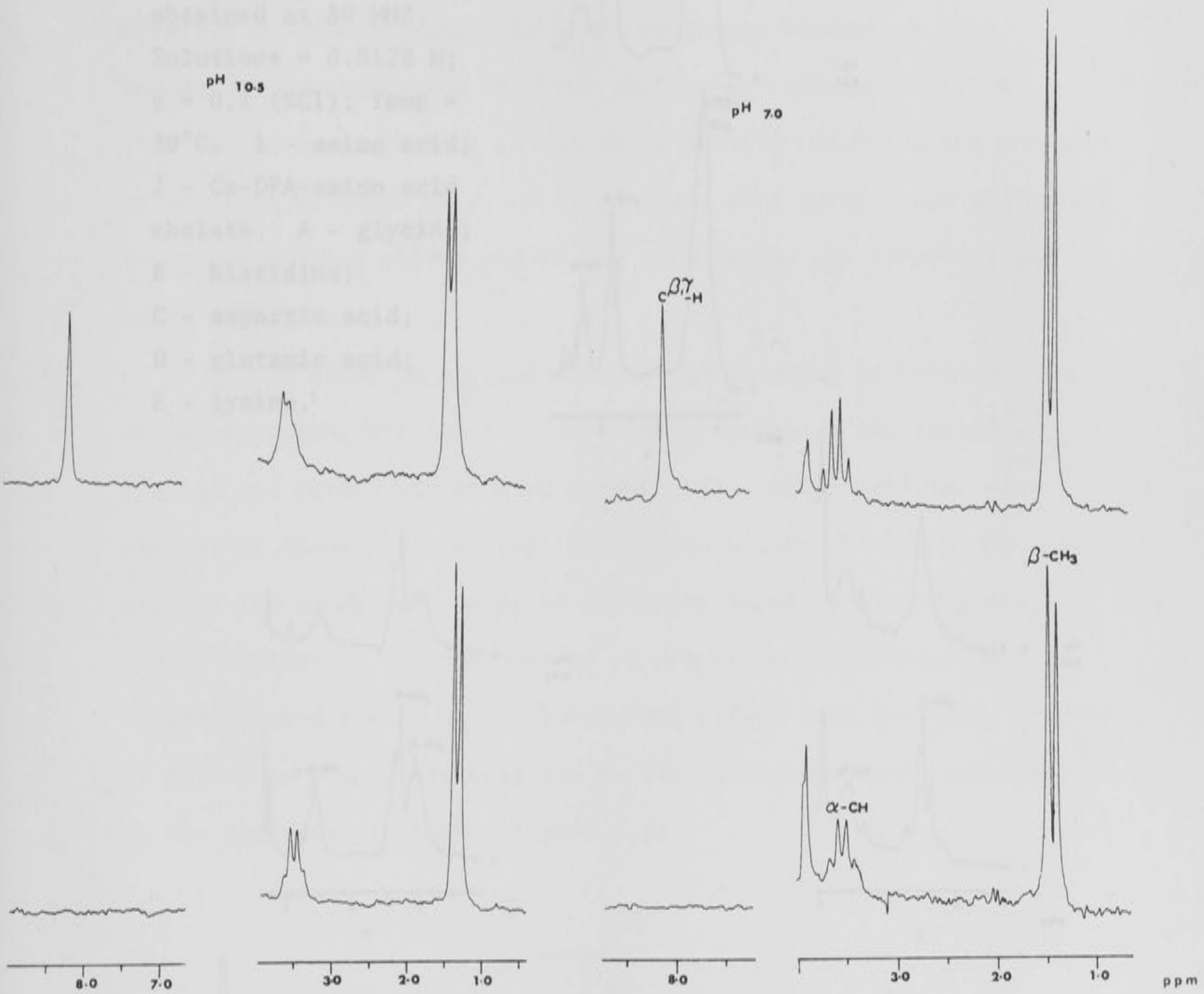
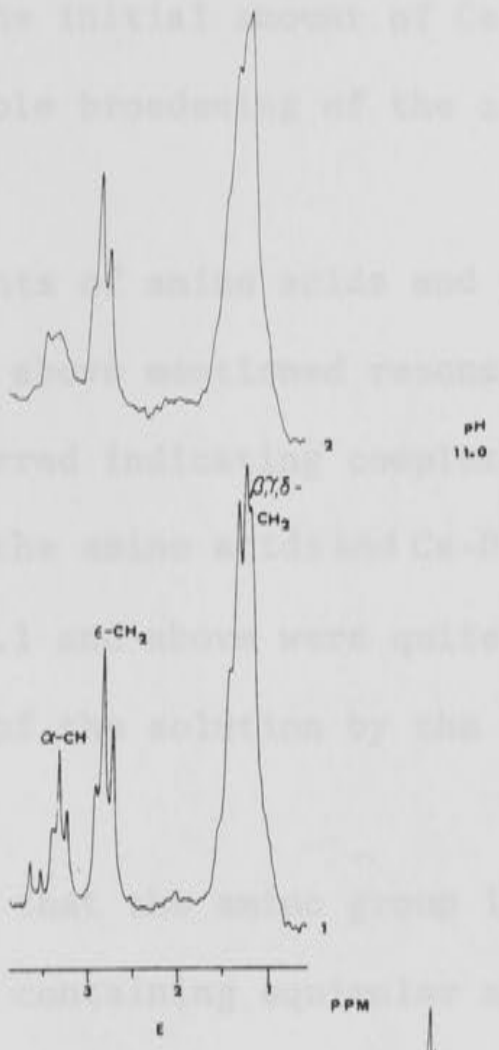
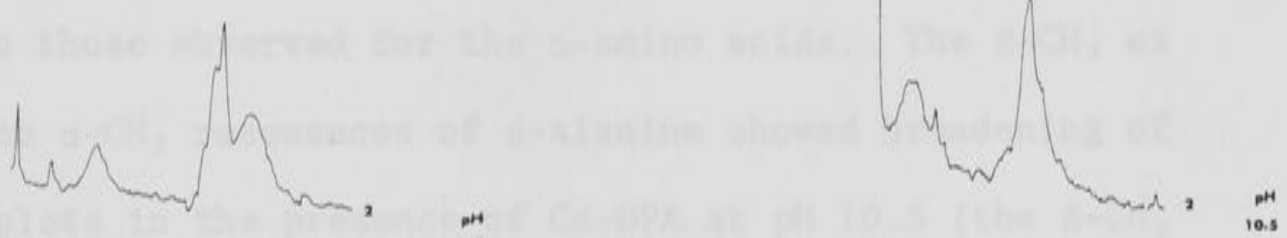
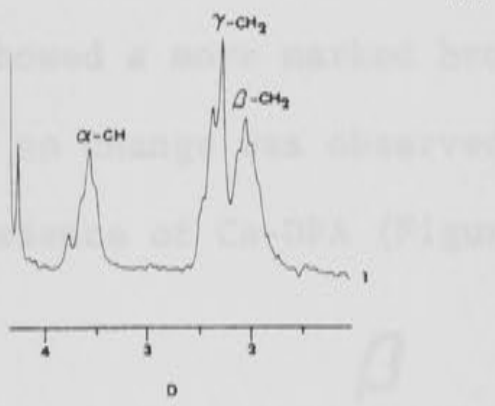


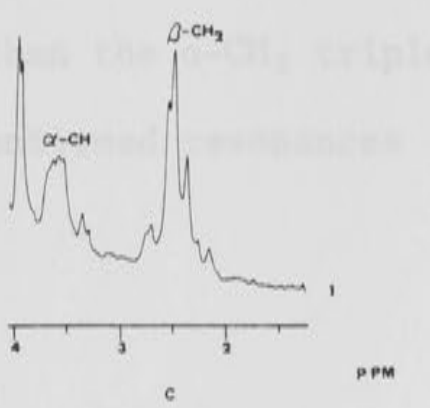
Figure 5.5a ^{1}H NMR spectra of alanine (lower trace) and Ca-DPA-alanine (upper trace) obtained at 80 MHz. Solutions were 0.0172 M. μ = 0.1 (KCl); Temp = 30°C.

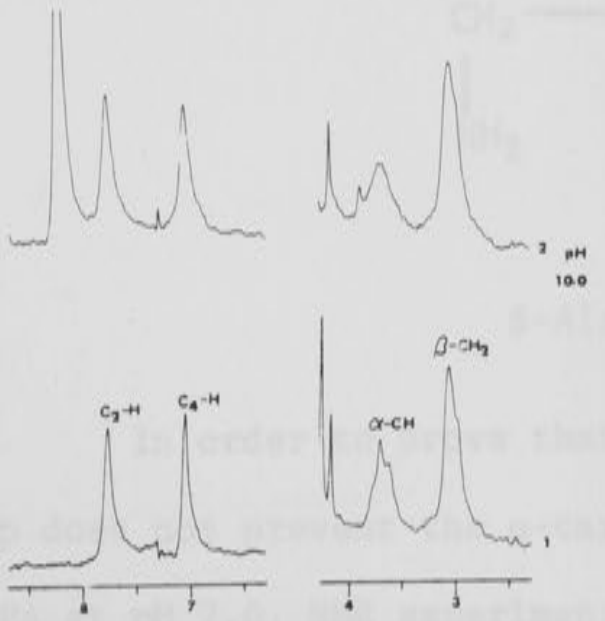
Figure 5.5b ^{1}H NMR spectra of some amino acids and Ca-DPA-amino acid chelates obtained at 80 MHZ. Solutions = 0.0172 M; μ = 0.1 (KCl); Temp = 30°C. 1 - amino acid; 2 - Ca-DPA-amino acid chelate. A - glycine; B - histidine; C - aspartic acid; D - glutamic acid; E - lysine.

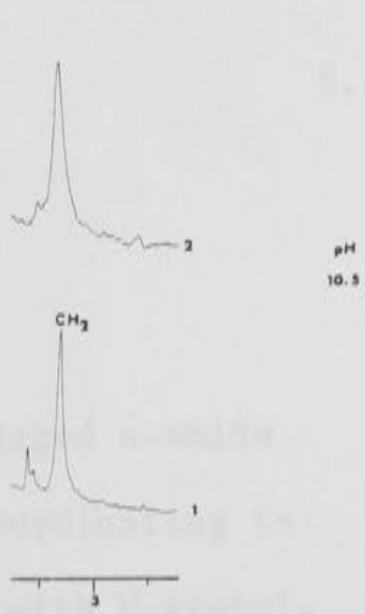








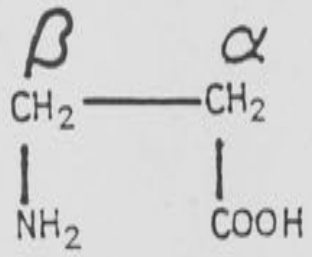




higher pH (≳ 10.0) but here the initial amount of Ca-DPA added was sufficient to cause a noticeable broadening of the α-CH and β-CH_2(CH_3) resonances.

When equimolar amounts of amino acids and Ca-DPA were mixed at alkaline pH (the pH where the above mentioned resonances were broadened) precipitation occurred indicating complex formation (the respective solutions of the amino acids and Ca-DPA in the presence of an ionic strength of μ = 0.1 and above were quite clear indicating that they were not salted out of the solution by the effect of the ionic strength).

In order to confirm that the amino group is involved in coordination, the solution containing equimolar amounts of β-alanine and Ca-DPA was studied by NMR. The result obtained was similar to those observed for the α-amino acids. The β-CH_2 as well as the α-CH_2 resonances of β-alanine showed broadening of their triplets in the presence of Ca-DPA at pH 10.5 (the β-CH_2 triplet showed a more marked broadening effect than the α-CH_2 triplet). At pH 7.0 no change was observed in the above mentioned resonances in the presence of Ca-DPA (Figure 5.6).



5.2

β-Alanine

In order to prove that the positively charged α-amino group does not prevent the α-carboxyl group from coordinating to Ca-DPA at pH 7.0, NMR experiments were carried out with N-acetylglycylglycine and N-acetylhistidine with Ca-DPA (Figure 5.7). As

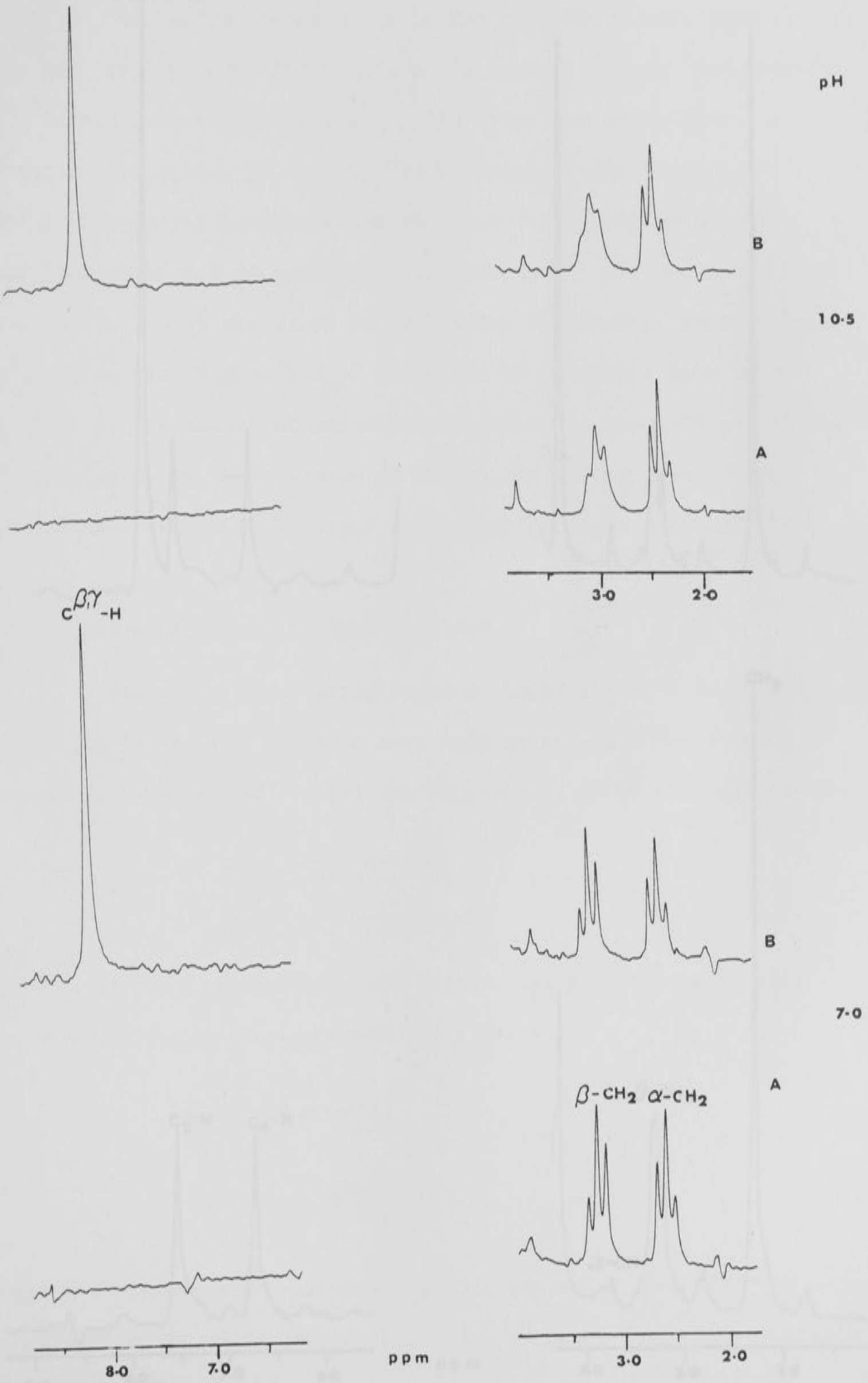


Figure 5.6 β-alanine-Ca-DPA studied at 80 MHz by ^{1}H NMR. A - free amino acid; B - Ca-DPA-amino acid chelate.

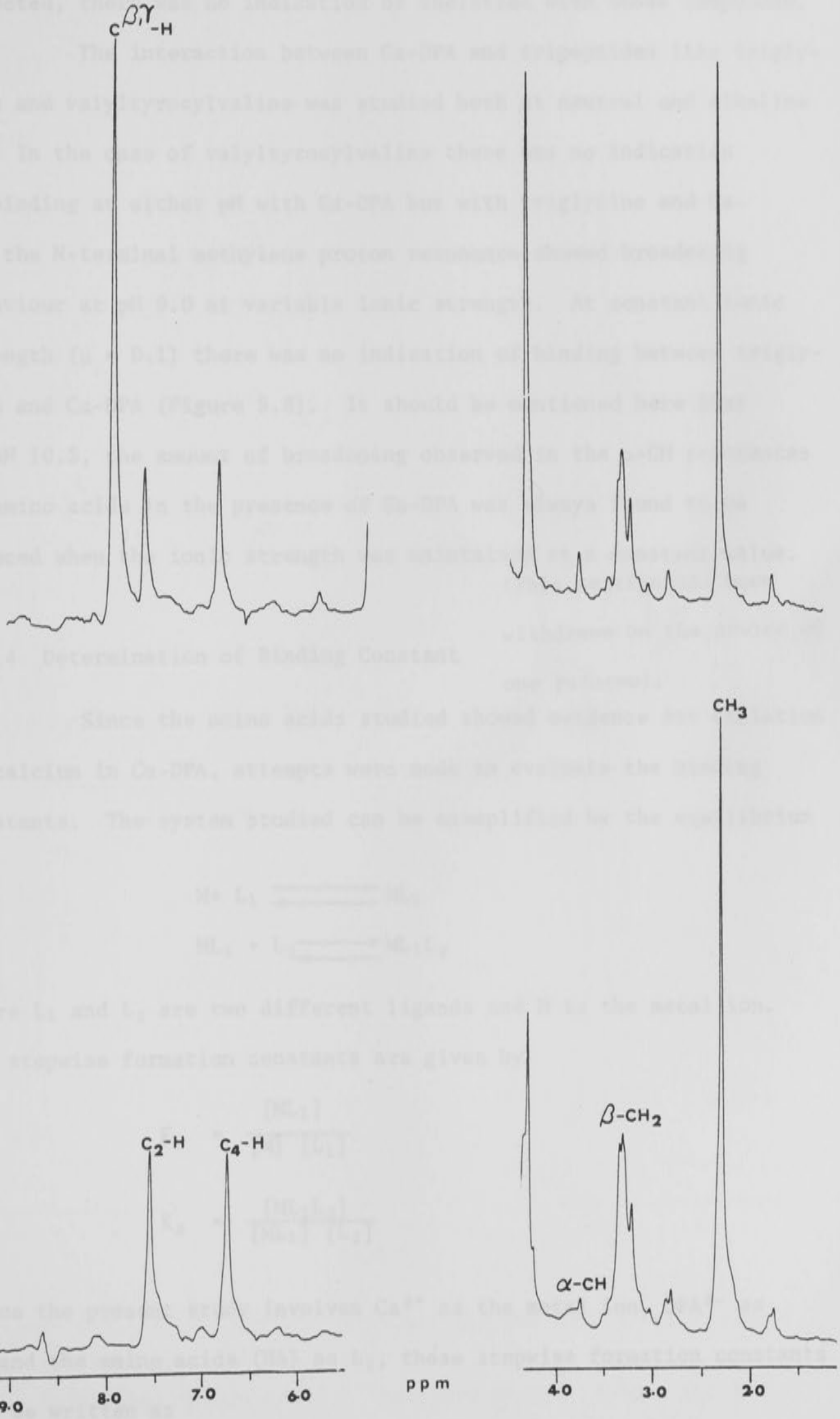


Figure 5.7 ^{1}H NMR spectra of N-acetyl histidine-Ca-DPA studied at 80 MHz. pH = 8.0.

expected, there was no indication of chelation with these compounds.

The interaction between Ca-DPA and tripeptides like triglycine and valyltyrosylvaline was studied both at neutral and alkaline pH. In the case of valyltyrosylvaline there was no indication of binding at either pH with Ca-DPA but with triglycine and Ca-DPA the N-terminal methylene proton resonance showed broadening behaviour at pH 9.0 at variable ionic strength. At constant ionic strength (μ = 0.1) there was no indication of binding between triglycine and Ca-DPA (Figure 5.8). It should be mentioned here that at pH 10.5, the amount of broadening observed in the α-CH resonances of amino acids in the presence of Ca-DPA was always found to be reduced when the ionic strength was maintained at a constant value.

5.3.4 Determination of Binding Constant

(This section has been withdrawn on the advice of one referee).

Since the amino acids studied showed evidence for chelation to calcium in Ca-DPA, attempts were made to evaluate the binding constants. The system studied can be exemplified by the equilibrium

$$M + L_1 \rightleftharpoons ML_1$$

$$ML_1 + L_2 \rightleftharpoons ML_1L_2$$

where L_1 and L_2 are two different ligands and M is the metal ion. The stepwise formation constants are given by

$$K_1 = \frac{[ML_1]}{[M]\ [L_1]}$$

$$K_2 = \frac{[ML_1L_2]}{[ML_1]\ [L_2]}$$

Since the present study involves Ca^{2+} as the metal ion, DPA^{2-} as L_1 and the amino acids (HA) as L_2, these stepwise formation constants can be written as

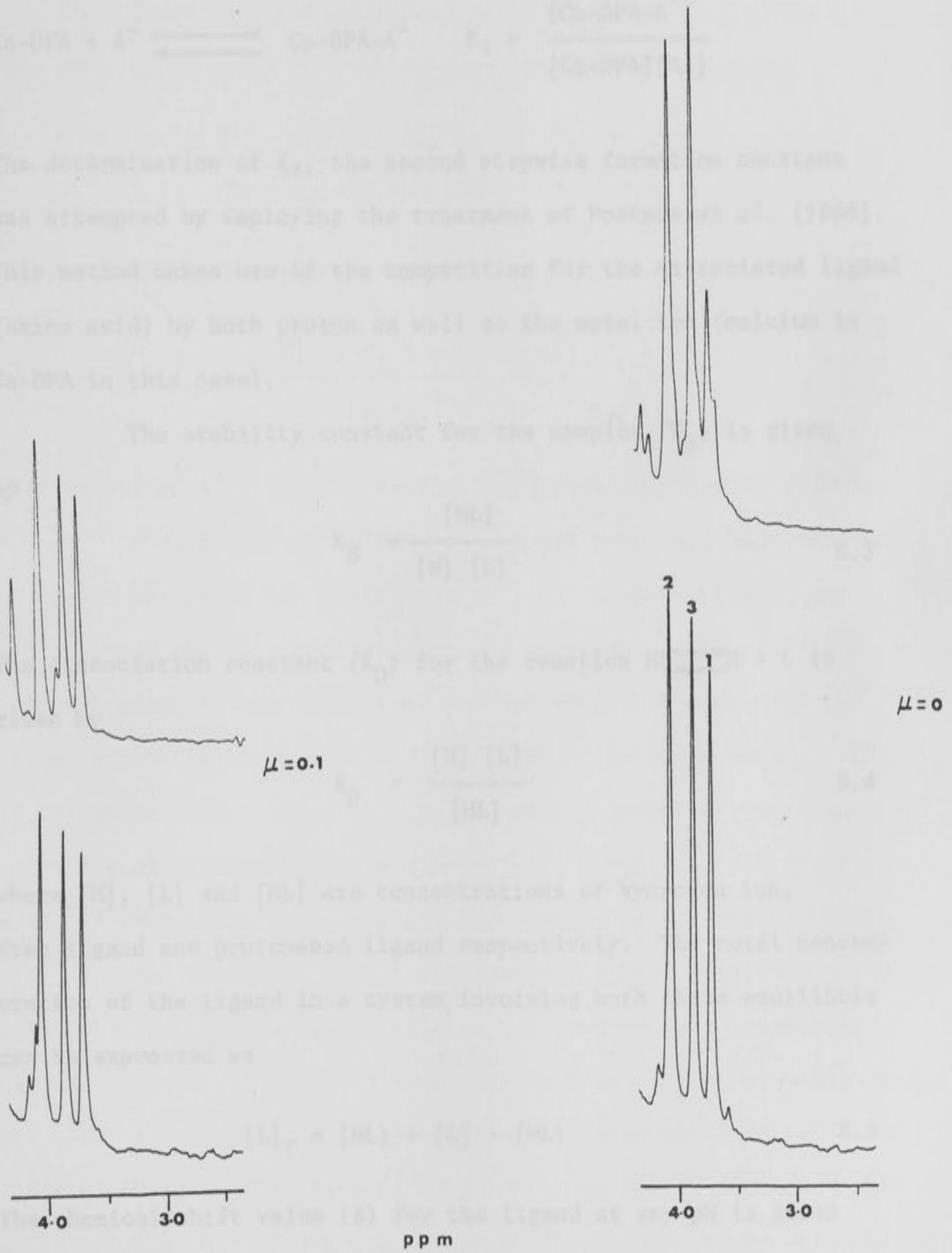


Figure 5.8 ^{1}H NMR spectra of triglycine and Ca-DPA-triglycine systems studied at 80 MHZ. pH = 8.5.
1 - N-terminal methylene, 2 - that from second residue and 3 - C-terminal methylene proton resonances.

$$Ca^{2+} + DPA^{2-} \rightleftharpoons Ca\text{-}DPA \qquad K_1 = \frac{[Ca\text{-}DPA]}{[Ca^{2+}]\ [DPA]}$$

$$Ca\text{-}DPA + A^{-} \rightleftharpoons Ca\text{-}DPA\text{-}A^{-} \qquad K_2 = \frac{[Ca\text{-}DPA\text{-}A^{-}]}{[Ca\text{-}DPA][A^{-}]}$$

The determination of K_2, the second stepwise formation constant was attempted by employing the treatment of Postmus *et al*. [1966]. This method makes use of the competition for the dissociated ligand (amino acid) by both proton as well as the metal ion (calcium in Ca-DPA in this case).

The stability constant for the complex (K_S) is given by

$$K_S = \frac{[ML]}{[M]\ [L]} \qquad 5.3$$

The dissociation constant (K_D) for the reaction $HL \rightleftharpoons H + L$ is given by

$$K_D = \frac{[H]\ [L]}{[HL]} \qquad 5.4$$

where [H], [L] and [HL] are concentrations of hydrogen ion, free ligand and protonated ligand respectively. The total concentration of the ligand in a system involving both these equilibria can be expressed as

$$[L]_T = [HL] + [L] + [ML] \qquad 5.5$$

The chemical shift value (δ) for the ligand at any pH is given by

$$\delta = \delta_{HL}[HL] + \delta_L\ [L] + \delta_{ML}\ [ML] \qquad 5.6$$

where δ_{HL} is the chemical shift of the ligand in the protonated

form, δ_L is the chemical shift of the ligand in the unprotonated form and δ_{ML} is the chemical shift of the complex.

Dividing equation 5.6 by equation 5.5 after expressing [ML] and [L] in terms of [HL] by equation 5.3 and 5.4 gives

$$\delta = \frac{\delta_{HL}[H] + \delta_L K_D + \delta_{ML} K_S K_D [M]}{[H] + K_D + K_S K_D [M]} \qquad 5.7$$

Rearranging this equation 5.7 gives

$$\delta = \delta_{ML} - \frac{\{[H] (\delta-\delta_{HL}) - K_D (\delta_L-\delta)\}}{K_S K_D [M]} \qquad 5.8$$

This equation (5.8) is used here to determine the binding constant.

When chelation occurs between the amino acid and calcium in Ca-DPA the pK of the α-amino as well as the α-carboxyl group can decrease due to coordination. The decrease in the pK of the carboxyl group of the amino acid was not studied because the pK of one of the carboxyl groups of DPA was in the same range as that of the amino acid. The pK of the α-carboxyl groups of the amino acids are approximately 2.0 and hence will complicate the study when Ca^{2+}-DPA-amino acid stystems are studied in the acidic range.

NMR titration series were carried out with the amino acids glycine, alanine and lysine in the presence and absence of Ca-DPA. The pKs of the α-amino groups were determined by monitoring the α-CH resonance of these amino acids and were found to be lower (≃0.1 pK unit) in the presence of Ca-DPA than in its absence (Table 5.1). In the case of alanine the pK of the α-amino group was also determined by monitoring the β-CH_3 resonance and found to be lower (≃0.2 pK unit) in the presence of Ca-DPA. The pK of the ε-amino group of lysine did not vary appreciably in the presence of Ca-DPA

In all the above cases the α-CH and the β-CH_2(β-CH_3) resonances of these amino acids in the presence of Ca-DPA broadened significantly near the pH corresponding to the dissociation of the α-amino group. Also, the titration curves for the above mentioned amino acids in the presence of Ca-DPA appeared to be more sigmoidal than those of the free amino acids consistent with cooperative binding between Ca-DPA and the amino acid.

The chemical shift value (δ) at each pH was plotted against $\frac{[H]\ (\delta-\delta_{HL}) - K_D\ (\delta_L-\delta)}{K_D\ [M]}$, where

K_D = dissociation constant of the ligand in the presence of Ca-DPA, and

[M] = concentration of Ca-DPA.

For the straight lines obtained, the intercept gives δ_{ML} and the slope, $-\frac{1}{K_S}$ from which the stability constant of the complex is calculated. The plot for alanine (α-CH resonance) is shown in Figure 5.9. The values of the binding constants obtained by this method are given in Table 5.1.

The values for these binding constants obtained by this method were found to be much higher than that reported by Tang *et al.*[1968]. This may be due to the lower ionic strength (μ = 0.1) employed in this present work than that (μ = 1.0) used by Tang *et al.* [1968]. However the higher binding constant values from this work should be treated with considerable caution since the observed differences in the pK values (≃0.1 pK unit) are only slightly greater than the experimental error (±0.05 pK unit) and hence the possibility of large percentage errors. The treatment of Postmus *et al.* [1966] does not invoke any major assumption and is fairly straight forward in its application. The error limits

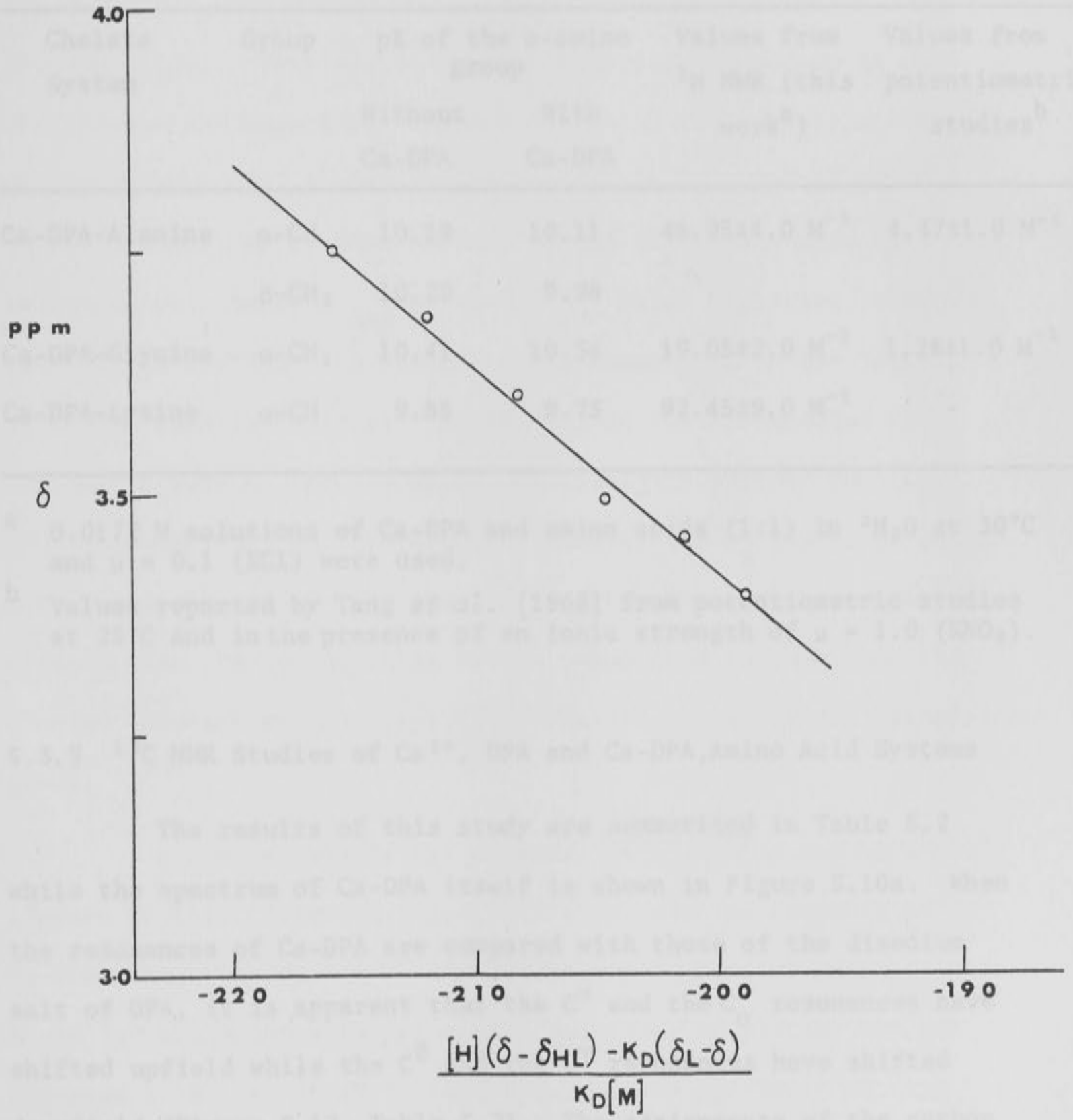


Figure 5.9 Determination of the stability constant for the Ca-DPA-alanine chelate. The data for the plot was obtained from the titration of the α-CH resonance of alanine.

for the values given in the horizontal axis of the plot (Figure 5.9) are in the range ±2.0 to 9.0.

TABLE 5.1

Stability constants of Ca-DPA-amino acid chelates

Chelate System	Group	pK of the α-amino group		Values from ^1H NMR (this work[a])	Values from potentiometric studies[b]
		Without Ca-DPA	With Ca-DPA		
Ca-DPA-Alanine	α-CH	10.19	10.11	46.95±4.0 M^{-1}	4.47±1.0 M^{-1}
	β-CH_3	10.20	9.98		
Ca-DPA-Glycine	α-CH_2	10.41	10.36	19.05±2.0 M^{-1}	1.29±1.0 M^{-1}
Ca-DPA-Lysine	α-CH	9.85	9.75	92.45±9.0 M^{-1}	-

[a] 0.0172 M solutions of Ca-DPA and amino acids (1:1) in ^2H_2O at 30°C and μ = 0.1 (KCl) were used.

[b] Values reported by Tang *et al.* [1968] from potentiometric studies at 25°C and in the presence of an ionic strength of μ = 1.0 (KNO_3).

5.3.5 ^{13}C NMR Studies of Ca^{2+}, DPA and Ca-DPA,Amino Acid Systems

The results of this study are summarized in Table 5.2 while the spectrum of Ca-DPA itself is shown in Figure 5.10a. When the resonances of Ca-DPA are compared with those of the disodium salt of DPA, it is apparent that the C^{α} and the C_o resonances have shifted upfield while the C^{β} and the C^{γ} resonances have shifted downfield (Figure 5.12, Table 5.2). The assignments of the carbon aromatic resonances of DPA are based on those of pyridine [Johnson and Jankowski 1972]. The ^{13}C spectra of alanine and Ca-DPA-alanine chelates are shown in Figure 5.10a. At both pH 7.0 and 10.5, the aromatic carbon and the carbonyl carbon resonances of the dipicolinic

acid moiety of Ca-DPA did not show any change at all (Table 5.2, Figure 5.10a), whereas the carbon resonances of alanine showed noticeable changes indicative of the chelation of alanine to calcium in Ca-DPA. At pH 7.0 the C_α and C_β resonances shifted downfield by 1.0 ppm and 0.6 ppm respectively. While the carbonyl carbon resonance of alanine was seen in the ^{13}C spectrum of the free amino acid itself, in the presence of Ca-DPA it was not detected at all (Figure 5.10a). This may be due to chelation to calcium in Ca-DPA through the negatively charged oxygen of the carboxylate group of the amino acid. A similar phenomenon was observed at pH 10.5 in the presence of Ca-DPA, whereas in the free amino acid this resonance was observed. Even with a 45° pulse and 4 sec. recycle time it was difficult to observe these resonances in the spectra of Ca-DPA-alanine chelates. At pH 10.5 in the presence of Ca-DPA, the C_α and C_β resonances of alanine again shifted downfield by 1.0 ppm and 1.85 ppm (Table 5.2). However the C_α resonance showed more broadening at pH 10.5 than at pH 7.0. These changes are consistent with the coordination of the amino acid through both the α-amino nitrogen and the negatively charged oxygen of the carboxylate group.

A similar result was obtained with glycine and Ca-DPA-glycine chelate (Figure 5.10b, Table 5.2). In the presence of Ca-DPA, the C_α resonance of glycine broadened more at pH 10.5 than at pH 7.0 while the carbonyl carbon resonance of glycine was not observed at all at pH 10.5. At pH 7.0 it was difficult to draw any conclusions regarding chelation because of the similarity in chemical shift of the carbonyl carbon resonances of glycine and DPA (Figure 5.10b).

The probable structures for the Ca-DPA-amino acid chelates

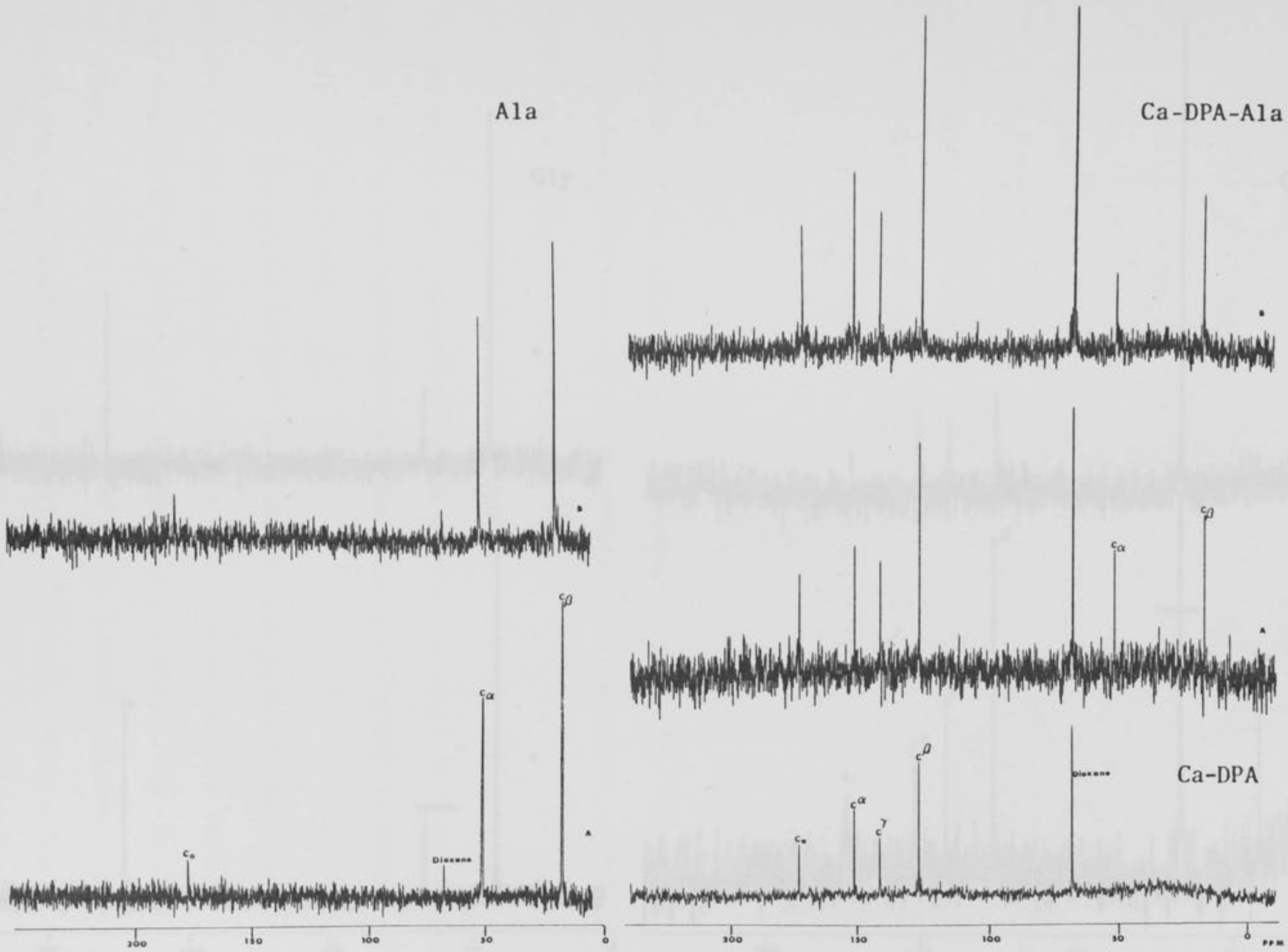


Figure 5.10a ^{13}C spectra of alanine and Ca-DPA-alanine. The spectra of the free amino acids were obtained with 0.56 M solutions at 20 MHz. Those for the complex and Ca-DPA with 0.0172 M solutions at 22.5 M MHz. The spectra of Ca-DPA was obtained at pH 8.0.

μ = 0.1 (KCl). Temperature = 30°C. A - pH 7.0 and B - pH 10.5

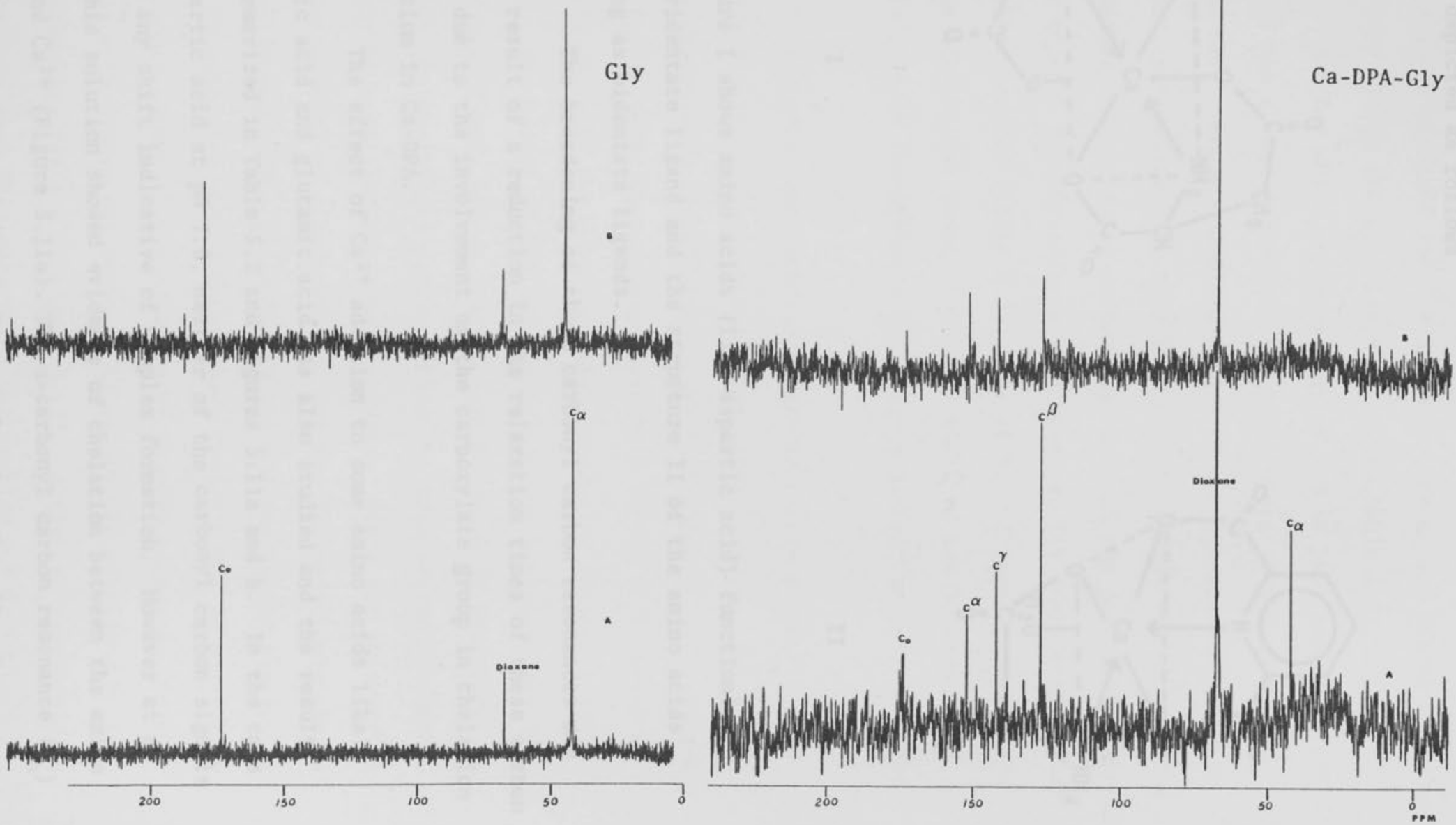
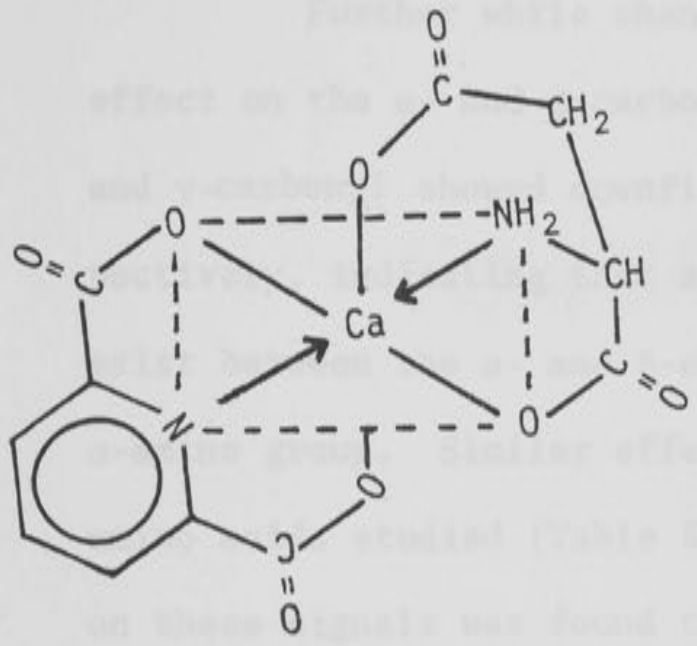


Figure 5.10b ^{13}C spectra of glycine and Ca-DPA-glycine. 0.67 M solution of the amino acid was used for obtaining the spectra at 20 MHz. The other details are the same as in Figure 5.10a.

can be depicted as follows



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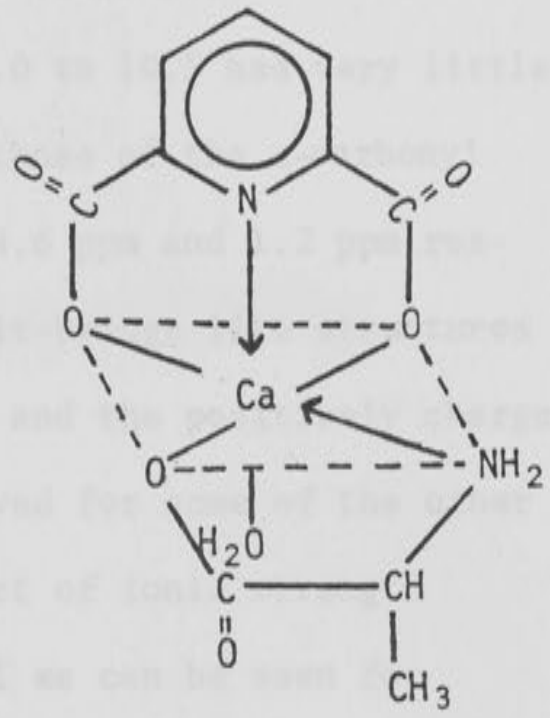


II

5.9

Structure I shows amino acids (like aspartic acid) functioning as a tridentate ligand and the structure II of the amino acids behaving as bidentate ligands.

The broadening of these carbonyl carbon resonances may be the result of a reduction in the relaxation times of these carbon nuclei due to the involvement of the carboxylate group in chelation to calcium in Ca-DPA.

The effect of Ca^{2+} addition to some amino acids like aspartic acid and glutamic acid was also studied and the results are summarized in Table 5.2 and Figures 5.11a and b. In the case of aspartic acid at pH 7.0, neither of the carbonyl carbon signals showed any shift indicative of complex formation. However at pH 10.5 this solution showed evidence of chelation between the amino acid and Ca^{2+} (Figure 5.11a). The α-carbonyl carbon resonance (C_o) shifted downfield by 3.0 ppm, the γ-carbonyl carbon ($C_{\gamma,o}$) resonance

showed a downfield shift of 1.4 ppm while the α- and β-carbon resonances showed very little change (Figure 5.11a and Table 5.2).

Further while changing from pH 7.0 to 10.5 had very little effect on the α- and β-carbon resonances, those of the α-carbonyl and γ-carbonyl showed downfield shifts of 4.6 ppm and 1.2 ppm respectively, indicating that some sort of salt-bridge like structures exist between the α- and β-carboxyl groups and the positively charged α-amino group. Similar effects were observed for some of the other amino acids studied (Table 5.2). The effect of ionic strength on these signals was found to be very small as can be seen for aspartic acid in the presence and absence of Ca^{2+} at pH 7.0 (Table 5.2).

A similar experiment with glutamic acid at pH 7.0 gave analogous results to that obtained for aspartic acid. But at pH 10.5 in thepresence of Ca^{2+} the δ-carbonyl carbon ($C_{\delta,o}$) resonance showed little shift whereas the α-carbonyl carbon (C_o) showed a downfield shift of 1.8 ppm along with broadening (Figure 5.11b).

The effect of DPA (disodium salt) on histidine was also studied by ^{13}C NMR. At pH 7.0 the α-amino group of histidine (pK 9.17) will be positively charged while the imidazolium nitrogen will only be partially positively charged. Hence any interaction (hydrogen bonding or electrostatic) between the negatively charged carboxyl groups of the DPA molecule and the positively charged groups of histidine should be apparent from ^{13}C NMR by monitoring the carbonyl carbon (C_o) resonances of the DPA molecule. However no noticeable change was observed in either the histidine or the DPA resonances (Table 5.2, Figure 5.12). A similar result was also obtained when this experiment was carried out at pH 10.5, Figure 5.12.

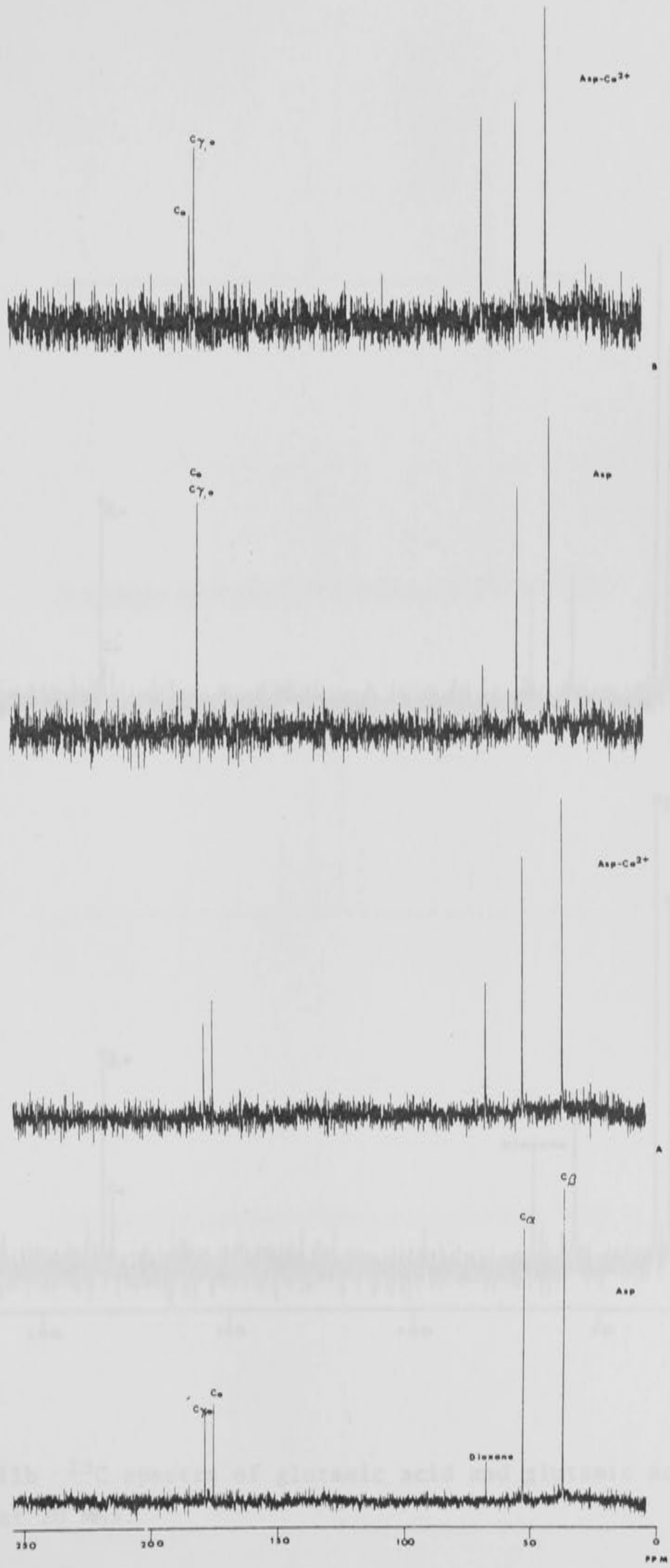


Figure **5.11a** ^{13}C spectra of aspartic acid and aspartic acid-Ca^{2+} studied at 20 MHz. Details in Table 5.2.
A - pH 7.0 and B - pH 10.5.

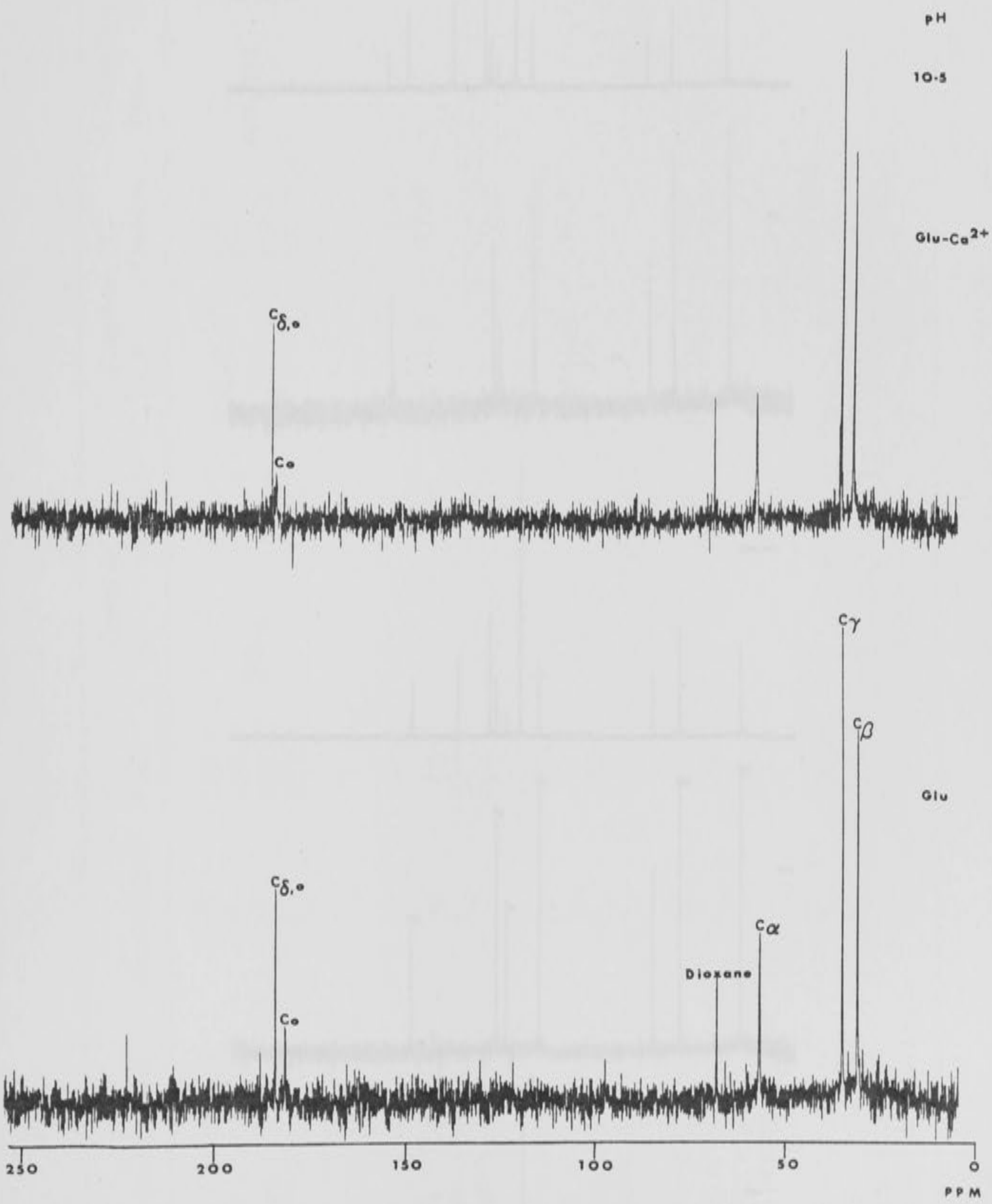


Figure **5.11b** ^{13}C spectra of glutamic acid and glutamic acid-Ca^{2+} obtained at 20 MHz.

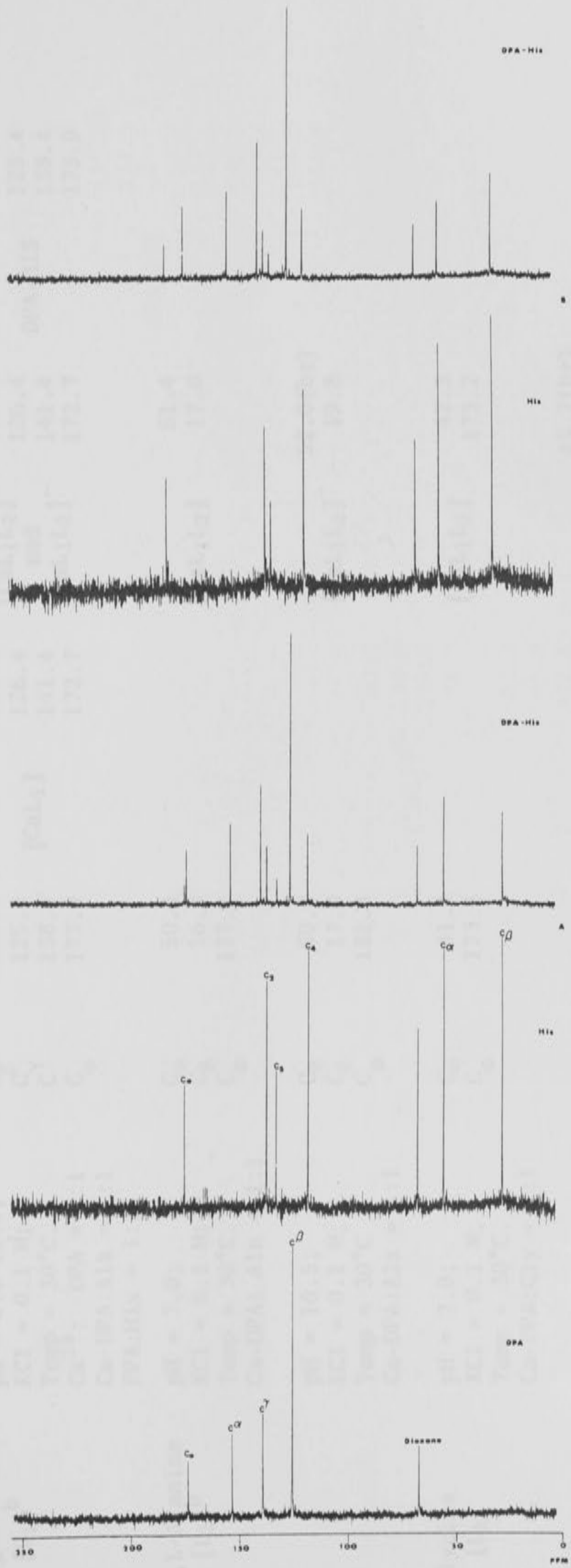


Figure **5.12** ^{13}C spectra of DPA-His studied at 20 MHz. Other details in Table 5.2.

A - pH 7.0, B - pH 10.5.

TABLE 5.2

^{13}C NMR studies of Ca^{2+}, DPA and Ca-DPA, amino acid systems[a]

System	Conditions	Resonances	Free	Complex I[c] Type	Complex I[c] Complex	Complex II[c] Type	Complex II[c] Complex	Complex III[c] Type	Complex III[c] Complex
DPA $[L_1]$[b]	pH = 6.0-11.0; KCl = 0.1 M; Temp = 30°C. Ca^{2+}: DPA = 1:1 Ca-DPA:Ala = 1:1 DPA:His = 1:1	C^{α}	153.4	$[CaL_1]$	151.4	$[CaL_1L_2]$[d] and $[CaL_1L_2]^-$	151.4	DPA-HIS	153.4
		C^{β}	125.4		126.4		126.4		125.4
		C^{γ}	138.4		141.4		141.4		138.4
		C_o	173.9		172.7		172.7		173.9
D,L-Alanine $[L_2]$[b]	pH = 7.0; KCl = 0.1 M; Temp = 30°C. Ca-DPA: Ala = 1:1	C_{α}	50.4				51.4		
		C_{β}	16.4			$[CaL_1L_2]$	17.0		
		C_o	177.4						
	pH = 10.5; KCl = 0.1 M; Temp = 30°C Ca-DPA:Ala = 1:1	C_{α}	50.9				52.0(br)		
		C_{β}	17.9			$[CaL_1L_2]^-$	19.8		
		C_o	181.4						
Glycine $[L_2]$	pH = 7.0; KCl = 0.1 M; Temp = 30°C. Ca-DPA:Gly = 1:1	C_{α}	41.4			$[CaL_1L_2]$	42.3		
		C_o	173.4				173.2		
	pH = 10.5; KCl = 0.1 M; Temp = 30°C. Ca-DPA:Ala = 1:1	C_{α}	42.9			$[CaL_1L_2]^-$	45.7(br)		
		C_o	178.7						

(continued)

TABLE 5.2 (continued)

System	Conditions	Resonances	Free	Complex I[c] Type	Complex I[c] Complex	Complex II[c] Type	Complex II[c] Complex	Complex III[c] Type	Complex III[c] Complex
L-Histidine	pH = 7.0;	C_α	55.4						55.4
$[L_2]$	KCl = 0.1 M;	C_β	28.4						28.4
	Temp = 30°C.	C_o	175.2						175.2
	DPA:His = 1:1	C_2	136.9						136.9
		C_4	117.6						117.6
		C_5	132.9						132.9
								DPA-HIS	
	pH = 10.5;	C_α	56.6						56.6
	KCl = 0.1 M;	C_β	32.1						32.1
	Temp = 30°C.	C_o	182.2						182.2
	DPA:His = 1:1	C_2	137.0						137.0
		C_4	118.8						118.8
		C_5	134.2						134.2
L-Aspartic acid	pH = 7.0;	C_α	52.4		52.7				
$[L_2]$	KCl = 0.1 M;	C_β	36.6	$[CaL_2]^+$	36.9				
	Temp + 30°C.	C_o	175.2		175.4				
	Ca^{2+}:Asp = 1:1	$C_{\gamma,o}$	178.6		178.7				
	pH = 10.5;	C_α	53.5		53.7				
	KCl = 0.1 M;	C_β	40.6	$[CaL_2]$	41.8				
	Temp = 30°C.	C_o	179.8		182.8				
	Ca^{2+}:Asp = 1:1	$C_{\gamma,o}$	179.8		181.2				

(continued)

TABLE 5.2 (continued)

System	Conditions	Resonances	Free	Complex I[c] Type	Complex I[c] Complex	Complex II[c] Type	Complex II[c] Complex	Complex III[c] Type	Complex III[c] Complex
D,L-Glutamic acid [L_2]	pH = 7.0; KCl = 0.1 M; Temp = 30°C Ca^{2+}:Glu = 1:1	C_α	54.1		54.8				
		C_β	26.4		27.1				
		C_γ	32.9	$[CaL_2]^+$	33.6				
		C_o	175.4		175.7				
		$C_{\delta,o}$	182.4		182.7				
	pH = 10.5; KCl = 0.1 M; Temp = 30°C. Ca^{2+}:Glu = 1:1	C_α	56.3		56.4				
		C_β	30.3		29.9				
		C_γ	34.3	$[CaL_2]$	32.9				
		C_o	181.4		183.2(br)				
		$C_{\delta,o}$	183.4		183.9				

a The ^{13}C resonances of amino acids were assigned based on those of Horsley *et al.* [1970], Allerhand *et al.* [1970] and Freedman *et al.* [1971]. The peaks were referenced from dioxane which was used as an internal standard and whose chemical shift value was taken as 67.4 ppm. All the chemical shift values given are in ppm. br = broad

b L_1 refers to DPA and L_2 refers to other amino acids

c Complex I represents Ca^{2+} complexes with amino acids and DPA; Complex II, Ca-DPA-amino acid and Complex III, DPA-amino acid complexes.

d $[CaL_1L_2]$ represents Ca-DPA-amino acid chelates at pH 7.0 and $[CaL_1L_2]^-$ that at pH 10.5.

5.3.6 Studies of Interactions Between Ribonuclease A and Ca^{2+}, DPA and Ca-DPA

Having established the nature of interactions between Ca-DPA and amino acids, NMR experiments were devised to study the interaction between Ca-DPA and proteins.

Ribonuclease A was the protein chosen since Ca-DPA has been found to stabilise this enzyme to heat (Appendix I). The effect of adding Ca-DPA to ribonuclease A was studied by using the high resolution NMR instrument (270 MHz). This experiment was carried out at both pH 6.0 and 10.5. At pH 6.0 no convincing evidence was obtained for the interaction of Ca-DPA with the various amino acid residues, but at pH 10.5 the ε-CH_2 resonance envelope of the lysine residues at 3.0 ppm showed splitting indicative of chelation between the ε-amino groups of the lysine residues and Ca-DPA. The α-CH resonance of Lys 1 at 3.40 ppm broadened in the presence of Ca-DPA and these effects are illustrated in Figure 5.13. Due to the low solubility of Ca-DPA in ^2H_2O (4 mg/ml) the enzyme solution became diluted as the Ca-DPA solution was added but molar ratios of Ca-DPA to enzyme of the order of 5 were reached.

The effect of dipicolinic acid (disodium salt) itself on ribonuclease A was studied at this pH (10.5) and the results are shown in Figure 5.14. Here the lysine ε-CH_2 resonance region remained unaffected. The increased splittings in the ε-CH_2 resonance envelope of lysine residues observed in Figure 5.14 as compared to that in both Figures 5.13 and 5.15 occur due to difference in instrumental resolution, the resolving power of the instrument being at its maximum for the series of spectra presented in Figure 5.14.

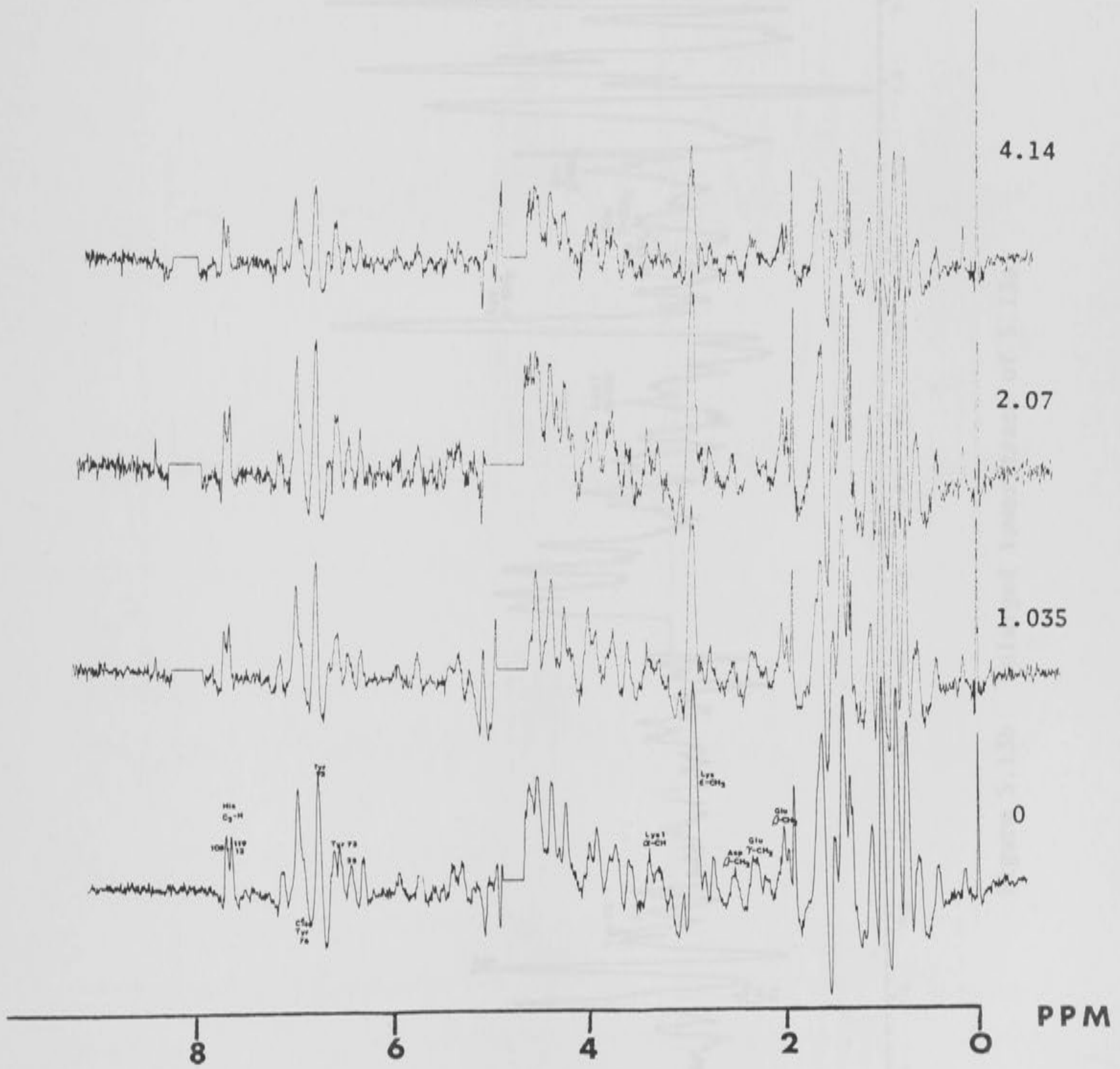


Figure 5.13a ^{1}H NMR spectra of RNase A-Ca-DPA system studied at 270 MHz. 5.8 mM of RNase A containing 0.1 M KCl at pH 10.5 and 20°C was employed. A stock solution of 0.0172 M Ca-DPA was prepared, from which the additions were made.

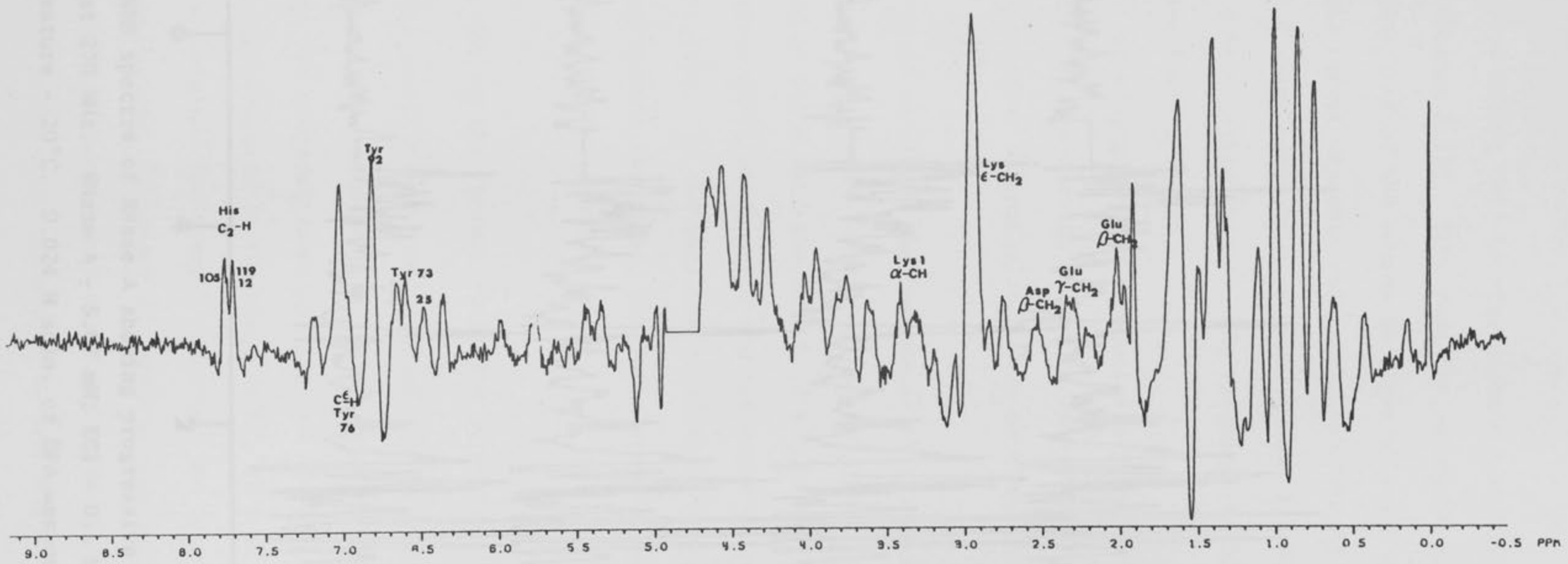


Figure 5.13b Enlarged lower trace of 5.13a.

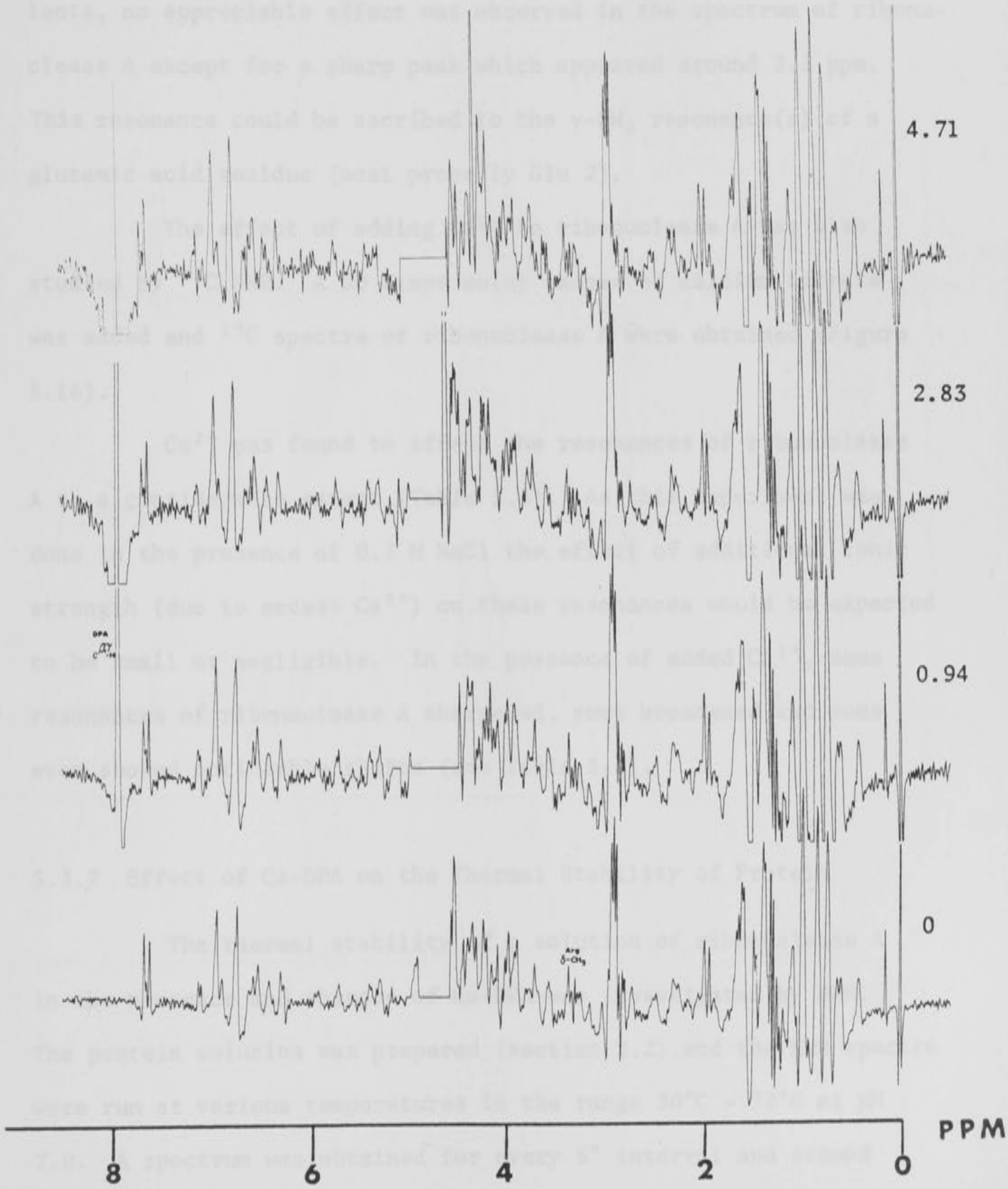


Figure 5.14 ^{1}H NMR spectra of RNase A showing progressive addition of DPA obtained at 270 MHz. RNase A - 5.07 mM; KCl - 0.1 M; pH - 10.5; Temperature - 20°C. 0.024 M soln. of DPA was used.

The effect of adding Ca^{2+} to ribonuclease A was also studied at pH 10.5 (Figure 5.15). Even when the amount of added Ca^{2+} was sixteen times that of the enzyme in terms of molar equivalents, no appreciable effect was observed in the spectrum of ribonuclease A except for a sharp peak which appeared around 2.3 ppm. This resonance could be ascribed to the γ-CH_2 resonance(s) of a glutamic acid residue (most probably Glu 2).

The effect of adding Ca^{2+} to ribonuclease A was also studied by ^{13}C NMR. A 50 times molar excess of calcium nitrate was added and ^{13}C spectra of ribonuclease A were obtained (Figure 5.16).

Ca^{2+} was found to affect the resonances of ribonuclease A to a considerable extent (Table 5.3). As this experiment was done in the presence of 0.3 M NaCl the effect of additional ionic strength (due to excess Ca^{2+}) on these resonances would be expected to be small or negligible. In the presence of added Ca^{2+}, some resonances of ribonuclease A sharpened, some broadened and some even showed noticeable shifts (see Table 5.3).

5.3.7 Effect of Ca-DPA on the Thermal Stability of Protein

The thermal stability of a solution of ribonuclease A in the presence and absence of Ca-DPA was investigated by NMR. The protein solution was prepared (section 2.2) and the NMR spectra were run at various temperatures in the range 30°C - 72°C at pH 7.0. A spectrum was obtained for every 5° interval and around the denaturation temperature, for every 2° interval. Under these experimental conditions, ribonuclease A was found to denature at approximately 61°C, in presence or absence of Ca-DPA (Figure 5.17).

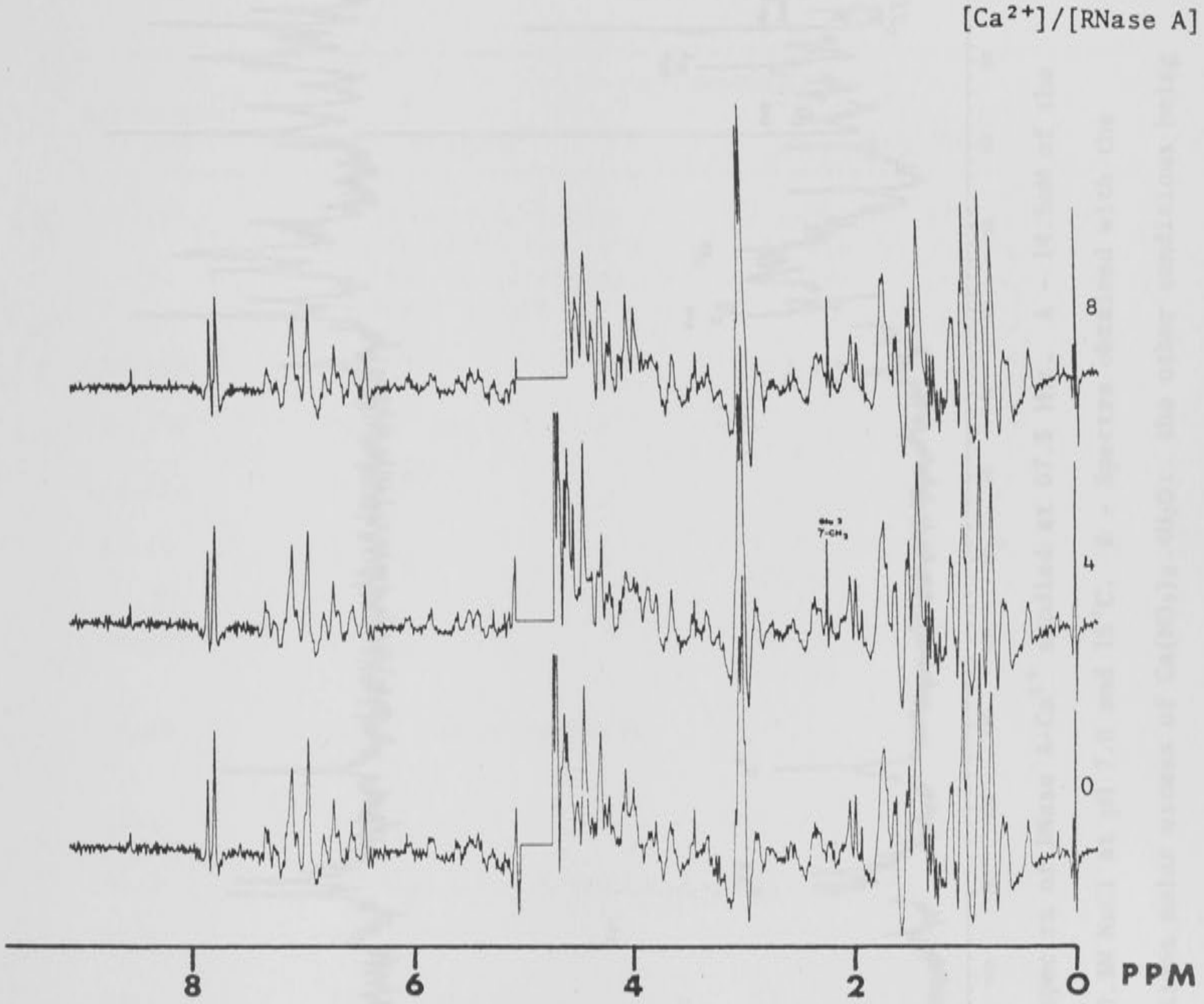


Figure 5.15 ^1H NMR study of RNase A-Ca^{2+} at 270 MHz. RNase A = 2.9 mM; KCl = 0.1 M; pH = 10.5; Temperature = 20°C. $Ca(NO_3)_2.4H_2O$ = 0.2 M stock solution.

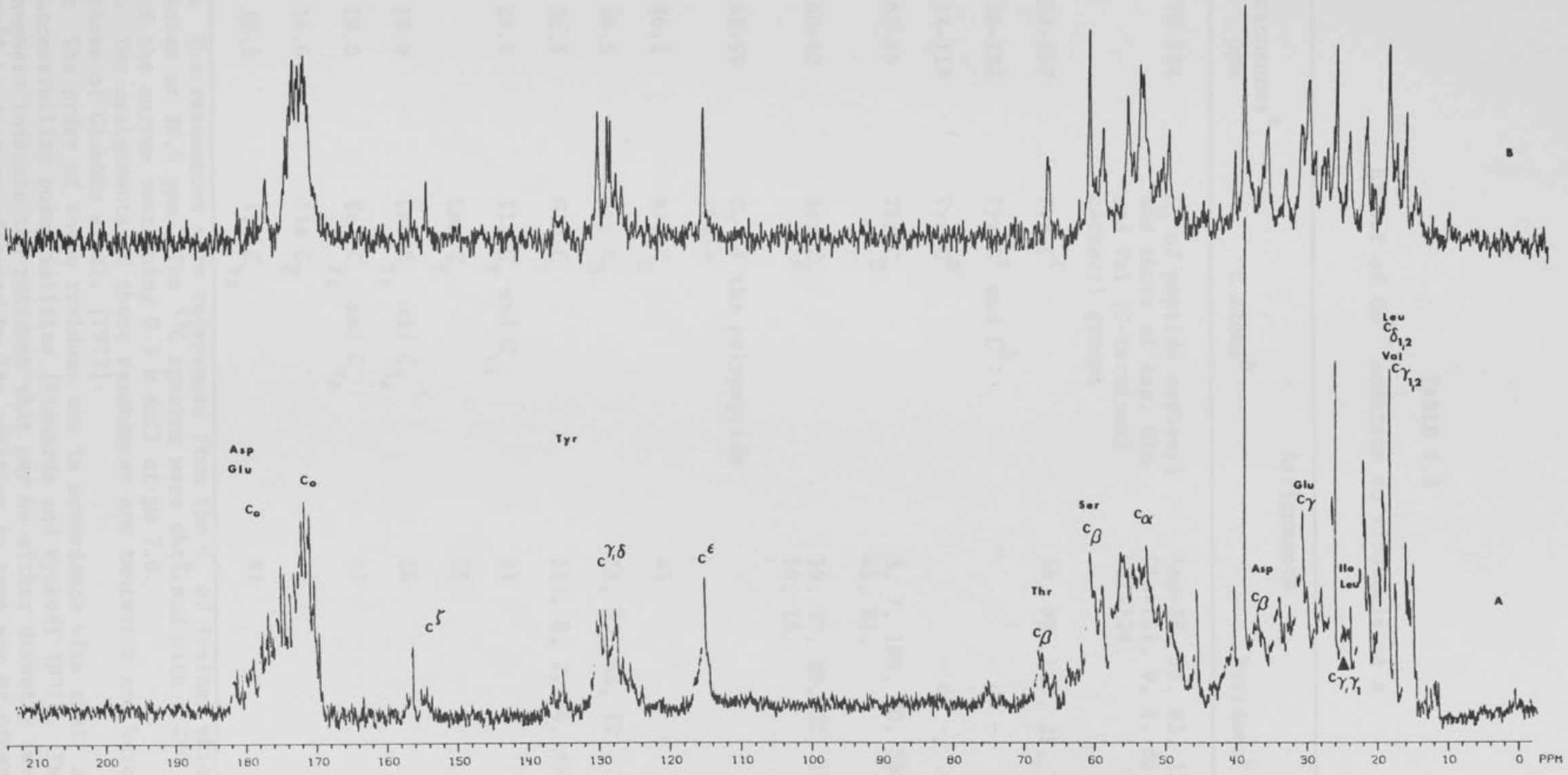


Figure 5.16 ^{13}C NMR spectra of RNase A-Ca^{2+} studied at 67.5 MHz. A - 14.5mM of the protein was used with 0.3M NaCl at pH 7.0 and 15°C. B - Spectra obtained with the protein containing 50 times molar excess of $Ca(NO_3)_2.4H_2O$: the other conditions being the same.

TABLE 5.3

Effect of Ca^{2+} addition to ribonuclease A

Resonances[a] ppm	Assignments: C atoms[b]	Assignments: Residues[c]
170-184	C_o of peptide carbonyl and those of Asp, Glu and Val (C-terminus) carboxyl groups	Asp-38, 53, 83, 121, 14. Glu-111, 9, 2, 86, 49. Val-124.
154-157	Tyr C^{ζ}	76, 92, 115, 25, 73.
126-132	Tyr C^{γ} and C^{δ}	- do -
114-118	Tyr C^{ε}	- do -
67-69	Thr C_{β}	3, 7, 100, 70, 78, 87, 99, 45, 82.
60-62	Ser C_{β}	59, 77, 89, 32, 123, 80, 50, 15.
48-59	C_{α} of the polypeptide chain	
46.1	Ala C_{α}	45
36.5	Asp C_{β}	38, 53, 83, 121, 14.
31.5	Glu C_{γ}	111, 9, 2, 86, 49.
24.5	Ile C_{γ} and C_{γ_1}	81
	Leu C_{γ}	35
19.9	Leu C_{γ_1} and C_{δ_2}	35
19.0	Val C_{γ_1} and C_{γ_2}	47
16.6	Ala C_{β}	45
15.5	Ile C_{γ_2}	81

a The resonances were referenced from the C_{ε} of lysines which was taken as 39.6 ppm. The ^{13}C spectra were obtained with a 20% solution of the enzyme containing 0.3 M NaCl at pH 7.0.
b The assignments of these resonances are tentative and based upon those of Glushko *et al.* [1972].
c The order of these residues are in accordance with their solvent accessibility potentialities [Richards and Wyckoff 1971]. The actual numbers indicate the residues that may be either directly involved in Ca^{2+} binding or affected by its addition in some way or other.

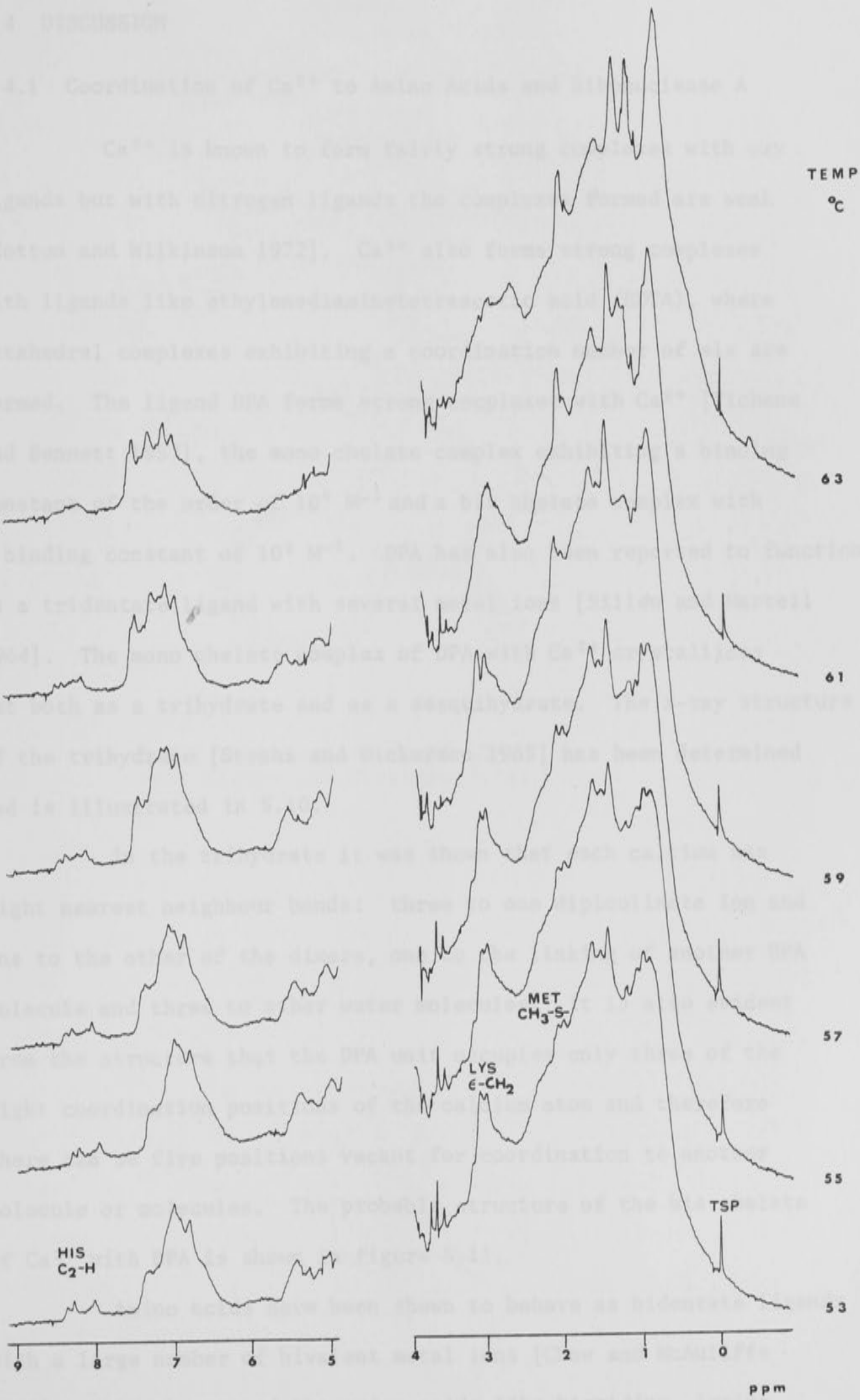


Figure 5.17 Thermal denaturation of RNase A studied at 80 MHz. RNase A - 7.25 mM; KCl - 0.1 M; pH - 7.0.

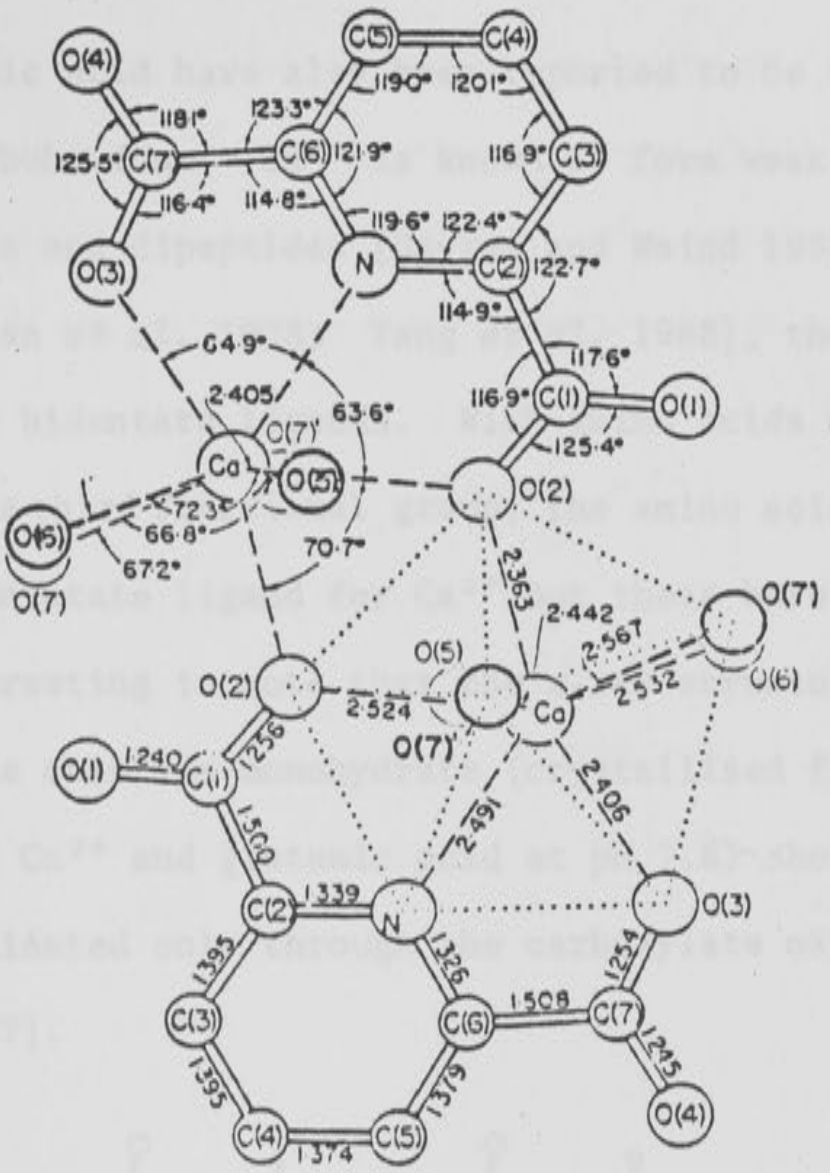
5.4 DISCUSSION

5.4.1 Coordination of Ca^{2+} to Amino Acids and Ribonuclease A

Ca^{2+} is known to form fairly strong complexes with oxy ligands but with nitrogen ligands the complexes formed are weak [Cotton and Wilkinson 1972]. Ca^{2+} also forms strong complexes with ligands like ethylenediaminetetraacetic acid (EDTA), where octahedral complexes exhibiting a coordination number of six are formed. The ligand DPA forms strong complexes with Ca^{2+} [Tichane and Bennett 1957], the mono chelate complex exhibiting a binding constant of the order of 10^4 M^{-1} and a bis chelate complex with a binding constant of 10^2 M^{-1}. DPA has also been reported to function as a tridentate ligand with several metal ions [Sillén and Martell 1964]. The mono chelate complex of DPA with Ca^{2+} crystallises out both as a trihydrate and as a sesquihydrate. The x-ray structure of the trihydrate [Strahs and Dickerson 1965] has been determined and is illustrated in 5.10.

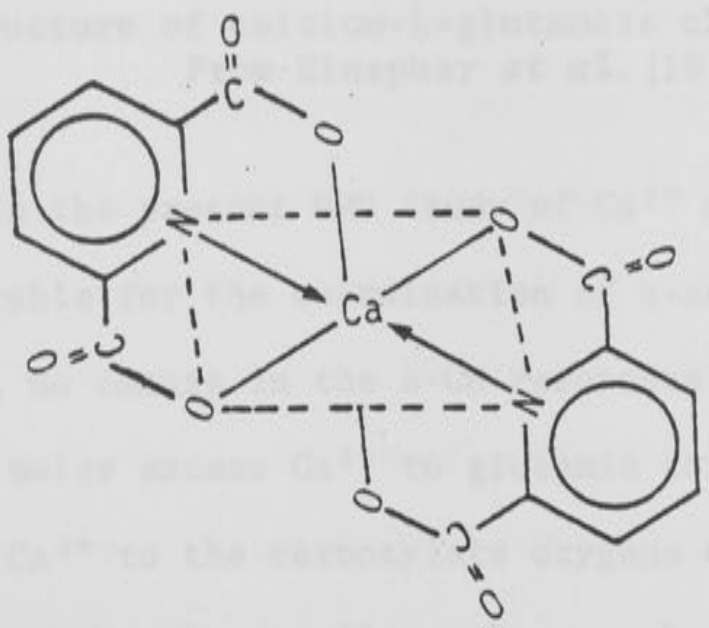
In the trihydrate it was shown that each calcium has eight nearest neighbour bonds: three to one dipicolinate ion and one to the other of the dimers, one to the linking of another DPA molecule and three to other water molecules. It is also evident from the structure that the DPA unit occupies only three of the eight coordination positions of the calcium atom and therefore there can be five positions vacant for coordination to another molecule or molecules. The probable structure of the bis chelate of Ca^{2+} with DPA is shown in figure 5.11.

Amino acids have been shown to behave as bidentate ligands with a large number of bivalent metal ions [Chow and McAuliffe 1975], although some of the amino acids like histidine, lysine



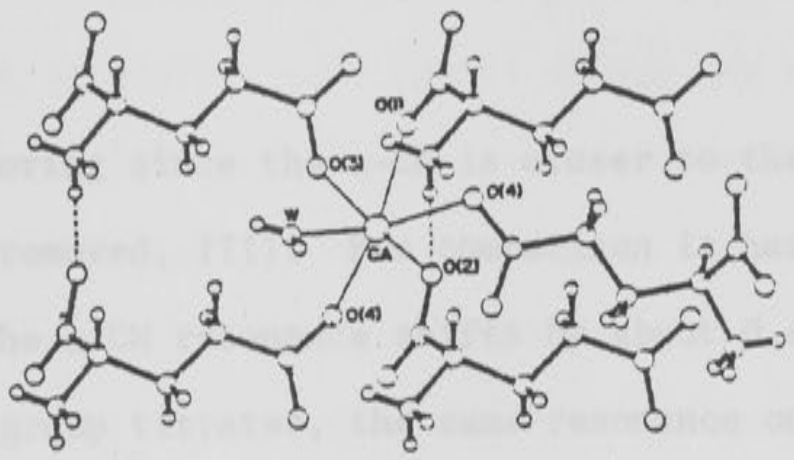
5.10

X-ray structure of Ca-DPA.$3H_2O$
From Strahs and Dickerson [1965]



5.11

and aspartic acid have also been reported to be tridentate in their chelation behaviour. Ca^{2+} is known to form weak complexes with amino acids and dipeptides [Davies and Waind 1950; Chung *et al.* 1971; Rajan *et al.* 1978; Tang *et al.* 1968], the amino acids functioning as bidentate ligands. With amino acids such as aspartic and having a third functional group, the amino acid can function as a tridentatate ligand for Ca^{2+} but these have rarely been reported. It is interesting to note that the x-ray structure of calcium-L-glutamate chloride monohydrate (crystallised from a solution containing Ca^{2+} and glutamic acid at pH 7.5) showed that amino acid coordinated only through the carboxylate oxygen [Einsphar *et al.* 1977].

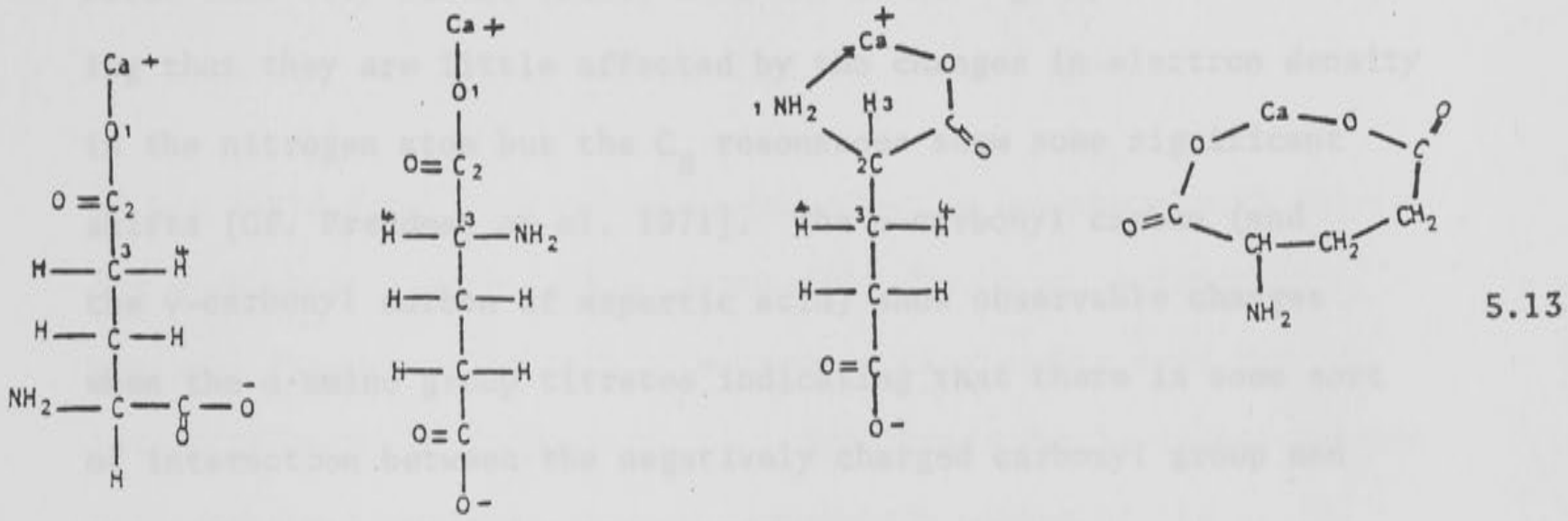


5.12

X-ray structure of calcium-L-glutamate chloride monohydrate
From Einsphar *et al.* [1977]

In the present NMR study of Ca^{2+} and glutamic acid at a pH favourable for the coordination of α-amino nitrogen of the amino acid, no change in the α-CH resonance was observed even when five times molar excess Ca^{2+} to glutamic acid was present. The binding of Ca^{2+} to the carboxylate oxygens of the glutamic acid (both the α-carboxyl as well as the γ-carboxyl) could not be observed by monitoring the α-CH and γ-CH_2 resonances. However Ca^{2+} is known to form only very weak complexes with glutamic acid (log K = 1.60,

Rajan *et al.* [1978]) and since the proton resonances monitored are four atoms removed from the carboxylate oxygens (the site of coordination) this above result is not surprising. The coordination of the amino acid through the nitrogen of the α-amino group would be more sensitive



I II III IV 5.13

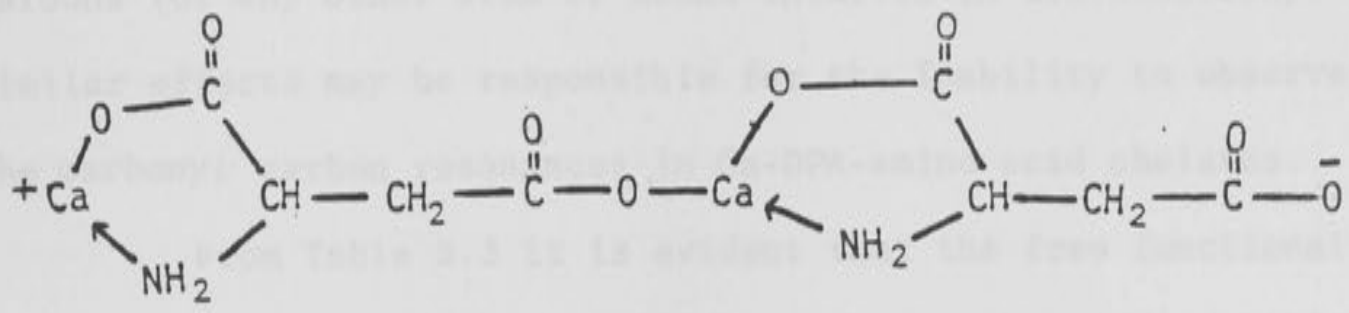
to NMR monitoring since the α-CH is closer to the donor atom (i.e. three atoms removed, III). For comparison it has been observed that while the α-CH resonance shifts by about 0.6 - 0.8 ppm when the α-amino group titrates, the same resonance only shows a shift of about 0.2 - 0.3 ppm when the α-carboxyl group titrates. Thus the α-CH resonance is less sensitive to change in the electron density of the α-carboxylate oxygen than on the α-nitrogen atom.

Even so no changes were observed in the α-CH resonance due to chelation to Ca^{2+}. However in the study of Ca^{2+} addition to ribonuclease A (Figure 5.15) the peak that appeared at 2.3 ppm could be attributed to Ca^{2+} binding to the negatively charged carboxylate oxygens of aspartic and glutamic acid residues in a protein molecule.

^{13}C NMR on the other hand was expected to be more informative since the sites of coordination, namely the negatively charged

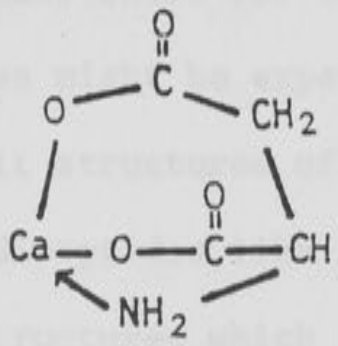
carboxylate oxygen (α- as well as side chain ones in some cases) and the α-amino nitrogen, are only two atoms away from the monitoring atom, that is the carbonyl carbon in the case of the former and the α-carbon in the case of the latter. The C_α resonances of amino acids show very little shifts when the α-amino groups titrate reflecting that they are little affected by the changes in electron density in the nitrogen atom but the C_β resonances show some significant shifts [Cf. Freedman *et al*. 1971]. The α-carbonyl carbon (and the γ-carbonyl carbon of aspartic acid) show observable changes when the α-amino group titrates indicating that there is some sort of interaction between the negatively charged carboxyl group and the positively charged α-amino group.

In the presence of Ca^{2+} at pH 7.0, the carbon resonances of aspartic acid exhibit very little change but at pH 10.5 there is a noticeable shift in both the α- and the γ-carbonyl carbon resonances of the aspartic acid indicating chelation of the type shown below. However from the magnitude of the downfield shifts it is obvious that the coordination of the β-carboxyl group is



5.14

weak. Structures of type shown below may not be favourable. In the case of glutamic acid at pH 10.5, the α-carbonyl carbon resonance shows significant downfield shift in the presence of Ca^{2+} whereas the δ-carbonyl was little affected indicating that the involvement



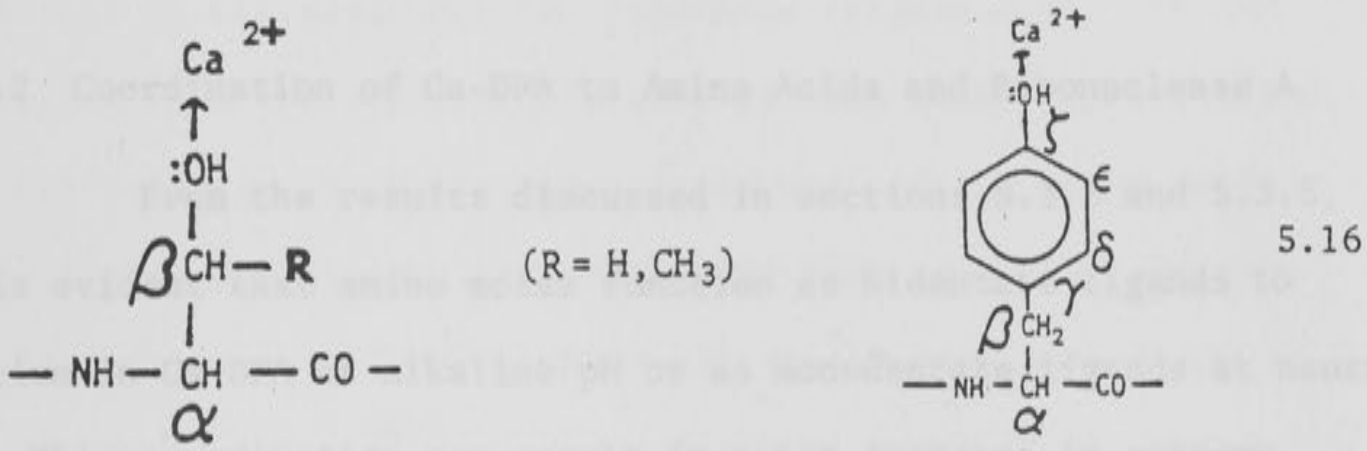
5.15

of the third functional group in coordination decreases with increasing side chain length. This is possibly due to an increase in motional freedom. The carbon resonances of glutamic acid were little affected by Ca^{2+} at pH 7.0. Weak bonds can be formed between Ca^{2+} and the negatively charged carboxylate oxygen of the α-, β-(Asp) and γ-carboxyl (Glu) groups (the amino acids functioning like monodentate ligands). Hence it would appear that complexes like the calcium-L-glutamate chloride monohydrate system could be expected to exist in solution as well.

In the case of glutamic acid, the α-carbonyl carbon resonance (C_o) broadened on chelation to Ca^{2+} (Figure 5.11b) again indicating that coordination does affect the relaxation behaviour of the carboxyl carbons (or any other atom or atoms involved in coordination). Similar effects may be responsible for the inability to observe the carbonyl carbon resonances in Ca-DPA-amino acid chelates.

From Table 5.3 it is evident that the free functional groups of some amino acid residues in ribonuclease A do exhibit interaction with Ca^{2+}. Residues which would be expected to exhibit this behaviour include the following: aspartic acid (five), glutamic acid (five), C-terminal valine (α-carboxyl group), serine (fifteen), threonine (ten) and tyrosine (six). Structures of the type I (p.167) can be visualised for the side chain carboxyl groups of aspartic

and glutamic acid residues while for the C-terminal valine α-carboxyl group,type II structures might be expected. Serine, threonine and tyrosine may exhibit structures of the type shown below. From studies with Ca^{2+} and dipeptides like glycylglycine, Davies and Waind [1950] proposed structures which involve the coordination



5.16

of peptide nitrogen as well. At pH 7.0 the α-amino group of Lys 1 is in the unprotonated form and hence can be expected to interact with Ca^{2+}, but it cannot be studied by monitoring the C_α resonance of that residue because (a) of the difficulty in singling out a resonance from a whole envelope of C_α resonances and (b) any change in electron density on the nitrogen due to chelation will have very little effect on the α-carbon. The interactions of the above mentioned amino acid residues of ribonuclease A with Ca^{2+} may alter the three-dimensional structure of the enzyme to some extent and some changes in the chemical shift values could be expected to result from this. That the enzyme does not undergo denaturation in the presence of Ca^{2+} was inferred from the ^1H NMR spectrum of the same sample. It can be seen from Figure 5.16 that the carbonyl carbon resonance region shows significant changes which probably arise from the interaction of Ca^{2+} with the carboxyl groups of the aspartic and glutamic acid residues (especially the downfield carbonyl carbon resonances from 178-184 ppm). Some of these resonances

broaden to a significant extent due to these interactions.

From the present ^{13}C NMR study it was apparent that there was little interaction between DPA (disodium salt) and histidine (α-amino acids) or with ribonuclease A as evidenced by the lack of any significant changes in the respective spectra.

5.4.2 Coordination of Ca-DPA to Amino Acids and Ribonuclease A

From the results discussed in sections 5.3.3 and 5.3.5, it is evident that amino acids function as bidentate ligands to calcium in Ca-DPA at alkaline pH or as monodentate ligands at neutral pH. This coordination may result in a net increase in entropy since water molecules coordinated to calcium in Ca-DPA are displaced by the chelation of amino acids and hence such a reaction may be a thermodynamically favourable one. The calcium ion in Ca-DPA is six coordinate, the complex having an octahedral geometry. The α-amino acids form five membered chelate rings by functioning as bidentate ligands (Structure II, p. 147). The involvement of the third functional group in the case of certain amino acids (tridentate ligands) like aspartic acid (Structure I, p.147) results in the formation of six membered chelate rings (and even larger rings with amino acids like lysine) which in general are less stable than the five membered ones. Of the amino acids studied in this work only lysine showed evidence for the involvement of the third functional group (ε-amino group) in chelation to calcium in Ca-DPA (Figure 5.5b).

For the α-amino acids and Ca-DPA at pH 7.0, the results suggest that the α-amino nitrogen is not involved in coordination but at pH ≥ 10.5, ^{1}H as well as ^{13}C NMR indicate that both the α-amino

nitrogen and the α-carboxylate oxygen are involved in coordination. The coordination of an amino group to calcium in Ca-DPA has been proved beyond doubt in the Ca-DPA-β-alanine chelate by the observation (at pH 10.5) of a greater effect for the β-CH_2 proton resonances of β-alanine than for the α-CH_2 resonances. At pH 7.0 Ca-DPA had no effect on the resonances of β-alanine (Figure 5.6). ^1H NMR indicated that the α-CH resonance of all the amino acids studied and the β-CH_2 (or β-CH_3) resonances of most of the amino acids exhibit changes (broadening) in the presence of Ca-DPA (section 5.3.3). From the ^{13}C NMR studies, it can be seen that at pH 10.5, the carbonyl carbon (C_o) of the α-carboxyl group of the amino acids exhibited broadening in the presence of Ca-DPA while the C_α and the C_β resonances show small shifts (1-2 ppm, Figures 5.10a and b, Table 5.2).

In contrast to the results mentioned in section 5.3.2 where the coordination of α-amino acids to Ca^{2+} is difficult to detect by ^1H NMR, that between the amino acids and calcium in Ca-DPA is evidenced by the broadening of the α-CH as well as the β-CH_2 (or β-CH_3) resonances of the amino acids (section 5.3.3). The ^{13}C NMR studies confirmed that the magnitude of the shifts and broadening was greater in the case of Ca-DPA and amino acids than with Ca^{2+} (section 5.3.5). From this it can be concluded that the calcium in Ca-DPA forms stronger complexes with the α-amino acids than does Ca^{2+} itself. However the binding constant values previously reported [Tang *et al.* 1968] for the Ca-DPA-amino acid chelates are much lower than those for the Ca^{2+}-amino acid chelates [Rajan *et al.* 1978]. In this context, the binding constant values obtained from the present work (section 5.3.4) are consistent with the above NMR results.

The evidence described in section 5.3.6 established that the amino acid residues present in ribonuclease A also coordinated to calcium in Ca-DPA. It was evident that the α-amino group of Lys 1 and the ε-amino groups of the lysine residues were involved in this binding.

Thermal denaturation experiments carried out with ribonuclease A and Ca-DPA monitored by NMR failed to show any heat stabilisation of this protein by Ca-DPA (section 5.3.8). This could possibly be due to the low solubility of Ca-DPA which precluded the use of higher concentration ratios of Ca-DPA to the enzyme for this experiment.

The broadening of resonances observed in the ^{1}H as well as ^{13}C NMR due to chelation of the amino acids to calcium in Ca-DPA could be due to various effects. One possibility is that intermediate rates of exchange between the coordinated and the non-coordinated forms of the ligand (amino acid) on the NMR time scale may lead to broadening of the resonances of the ligand. This proposal can be tested by lowering or raising the temperature and studying the complex formation at those temperatures. Below a certain temperature the rate of exchange will be sufficiently slow so that two signals will be observed, one corresponding to the complex and another to the free amino acid itself. Alternatively, at higher temperatures the rate of exchange will increase so that a single resonance with a different chemical shift value would be seen. Alternatively, broadening may be due to the contribution from the dipolar interactions [Bramley and Johnson 1971] on the spin-lattice relaxation time (T_1) which will lead to a higher value of $\nu_{\frac{1}{2}}$, the spectral width at half height (5.11) and hence a broad signal.

$$\nu_{\frac{1}{2}} = \frac{1}{\pi T_1} \qquad 5.17$$

In the case of weak complexation like that involved in this study, an increase of temperature may enhance the thermal decomposition of the complex and hence may not be conclusive in establishing the intermediate rate of exchange as a cause for broadening. This problem may well be settled by carrying out this experiment at lower temperatures, where the resolution of the broad signal into two signals would establish whether the phenomenon mentioned above is responsible for the observed broadening.

5.5 CONCLUSIONS

The foregoing NMR investigation has established that amino acids and ribonuclease A form weak complexes with Ca^{2+} and Ca-DPA. Further it was apparent that the calcium in Ca-DPA forms stronger complexes with amino acids and ribonuclease A than does Ca^{2+} itself.

Hence the presence of comparatively large proportions of Ca^{2+} and DPA in bacterial spores could be responsible for their stability to heat. The proteins (and enzymes) and other biological molecules present in bacterial spores could be rendered heat stable due to chelation to calcium in Ca-DPA.

APPENDIX I

APPENDIX I

HEAT STABILISATION OF ENZYMES BY Ca-DPA

Summary of the Heating Experiments

Enzyme System	Experiment	Experimental conditions	Results: Enzyme Activity	Enzyme % of Activity	Enzyme (heated) Activity	Enzyme (heated) % of Activity	Enzyme + Ca-DPA Activity	Enzyme + Ca-DPA % of Activity	Enzyme + Ca-DPA (heated) Activity	Enzyme + Ca-DPA (heated) % of Activity	Conclusions
Lysozyme	I	Sample from Brendon Hammer (Repetition of his experiment). Temperature = 130°C; Duration = 21 hours; Heating in test-tubes.	4.38	100	0.38	9	3.67	100	1.0	27	About 15% heat stabilisation
	II	Lysozyme lyophilized thrice from distilled water. Lysozyme + Ca-DPA mixture (1:3) lyophilized twice from distilled water. Temperature = 130°C; Duration = 21 hours; Heating in test-tubes.	4.93	100	0.10	2	5.76	100	0.23	4	No satisfactory indication of heat stabilisation
	III	Above experiment was repeated.	4.93	100	0.10	2	9.75	100	1.8	18	About 15% heat stabilisation
	IV	Samples from experiment II. Equilibrated; Sealed tubes; Temperature = 130°C; Duration = 21 hours.	4.18	100	0.015	0.35	9.03	100	0.29	3.2	No satisfactory Indication of heat stabilisation
	V	Above experiment was repeated.	9.10	100	0.53	5.8	9.18	100	1.1	12	-do-

(continued)

APPENDIX I (continued)

Enzyme System	Experiment	Experimental conditions	Results: Enzyme Activity	Enzyme % of Activity	Enzyme (heated) Activity	Enzyme (heated) % of Activity	Enzyme + Ca-DPA Activity	Enzyme + Ca-DPA % of Activity	Enzyme + Ca-DPA (heated) Activity	Enzyme + Ca-DPA (heated) % of Activity	Conclusions
Lysozyme	VI	Samples from experiment II. Equilibrated; Sealed tubes; Temperature = 120°C; Duration = 21 hours.	15.92	100	5.11	32	13.87	100	5.51	40	No satisfactory indication of heat stabilisation.
	VII	Both the enzyme and the Ca-DPA treated enzyme were lyophilized once from distilled water. Equilibrated; Sealed tubes; Temperature = 120°C; Duration = 21 hours.	15.9	100	4.72	30	14.5	100	5.31	37	-do-
Ribonuclease A	I	RNase A - lyophilized thrice from distilled water. Ca-DPA treated RNase A - lyophilized once from distilled water. Equilibrated; Sealed tubes; Temperature = 100°C; Duration = 16 hours.	0.84	100	0.83	99	0.81	100	0.78	96	No satisfactory indication of heat stabilisation.
	II	Samples from I. Heating in test-tubes; Temperature = 130°C; Duration = 21 hours.	0.60	100	0	0	0.72	100	0.52	72	Very high heat stabilisation.

(continued)

APPENDIX I (continued)

Enzyme System	Experiment	Experimental conditions	Results: Enzyme Activity	Enzyme % of Activity	Enzyme (heated) Activity	Enzyme (heated) % of Activity	Enzyme + Ca-DPA Activity	Enzyme + Ca-DPA % of Activity	Enzyme + Ca-DPA (heated) Activity	Enzyme + Ca-DPA (heated) % of Activity	Conclusions
Ribonuclease A	III	Samples from I. Equilibrated; Sealed tubes; Temperature = 130°C; Duration = 21 hours.	0.69	100	0	0	0.70	100	0.28	40	About 40% heat stabilisation
	IV	Both the enzyme and the Ca-DPA treated enzyme were lyophilized once from distilled water. Equilibrated; sealed tubes; Temperature = 110°C; Duration = 21 hours.	0.76	100	0.68	90	0.79	100	0.76	96	No satisfactory indication of heat stabilisation
	V	Samples from IV. Equilibrated; sealed tubes; Temperature = 120°C; Duration = 21 hours.	0.63	100	0.41	66	0.65	100	0.58	90	About 20% heat stabilisation
	VI	Samples from IV. Equilibrated; Sealed tubes; Temperature = 140°C; Duration = 21 hours.	0.63	100	0	0	0.65	100	0.35	54	Substantial heat stabilisation
Lactic dehydrogenase	I	Equilibrated at 4°C; Sealed tubes; Temperature = 80°C; Duration = 20 hours.	285	100	0	0	87	100	0	0	No indication of heat stabilisation

(continued)

APPENDIX I (continued)

Enzyme System	Experiment	Experimental conditions	Results: Enzyme Activity	Enzyme % of Activity	Enzyme (heated) Activity	Enzyme (heated) % of Activity	Enzyme + Ca-DPA Activity	Enzyme + Ca-DPA % of Activity	Enzyme + Ca-DPA (heated) Activity	Enzyme + Ca-DPA (heated) % of Activity	Conclusions
Lactic dehydrogenase	II	Equilibrated at 4°C; Sealed tubes; Temperature = 60°C; Duration = 20.5 hours.	101	100	0	0	746	100	352	47	About 50% heat stabilisation
Malic dehydrogenase	I	Equilibrated at 4°C; Sealed tubes; Temperature = 80°C; Duration = 20 hours.	2627	100	0	0	609	100	0	0	No indication of heat stabilisation
	II	Equilibrated at 4°C; Sealed tubes; Temperature = 60°C; Duration = 20 hours.	145	100	0	0	177	100	13	7	No satisfactory indication of heat stabilisation
	III	Equilibrated at 4°C; Sealed tubes; Temperature = 50°C; Duration = 20 hours.	899	100	280	31	1644	100	462	28	-do-
Glutamate-oxaloacetate transaminase		Equilibrated at 4°C; Sealed tubes; Temperature = 80°C; Duration = 20 hours.	888	100	17.5	2	506	100	12.5	2.5	No satisfactory indication of heat stabilisation

EXPERIMENTAL CONDITIONS

With enzymes an enzyme-Ca-DPA complex of ratio 1:3 (by weight) was prepared. A solution of 4 mg/ml of Ca-DPA in water was prepared by dissolving Ca-DPA in warm distilled water. To a solution containing 60 mg of Ca-DPA in water, 20 mg of enzyme was added. The clear solution was then lyophilized. Initially the once lyophilized complex was then dissolved in the minimum amount of water and then warmed slightly to get a clear solution and then lyophilized again. This is to ensure maximum homogeneity of the enzyme-Ca-DPA complex. Later, these lyophilizations were done only once. All the enzymes used were lyophilized at least once from distilled water.

Enzymes like lactic dehydrogenase, malic dehydrogenase and glutamate-oxaloacetate transaminase (GOT) are commercially obtained as suspensions in aqueous solution containing 2-3 M ammonium sulphate. The enzyme-Ca-DPA complex (1:3) was prepared by adding appropriate amounts of Ca-DPA solution to the enzyme suspension and then carefully evaporating in tubes used for heating experiments (see below). The same treatment was employed for the enzyme suspension as well.

About sufficient amount of enzyme was weighed out into a clean dry test-tube and covered with a piece of aluminium foil. The same thing was done with the complex also. The test-tubes were heated by placing them in an oven maintained at the appropriate temperature for the specified time.

For all the enzyme systems studied heating experiments were also done by heating the samples in tubes equilibrated under constant

humidity conditions for sometime. These experiments were done with tubes 10 cm in length; 0.5 cm, inner diameter; 0.15 cm, thickness and with a volume of 2 ml . Two such tubes with known amounts of the lyophilized enzyme and two other tubes with known amounts of the enzyme-Ca-DPA complex were taken. Hence, each enzyme system involved the use of four such tubes. All the four tubes were kept in a cylindrical glass vessel with a stopper having a provision for being connected to a vacuum line. A beaker containing saturated solution of magnesium nitrate ($Mg(NO_3)_2.6H_2O$) was kept in the assembly to provide a relative humidity of 50-52%. The beaker was covered with a tissue paper to prevent the water from splashing when the cylindrical vessel was evacuated. Also cotton plugs were used to cover the top of the tubes to prevent that water from entering the tubes. The whole assembly was then connected to a vacuum line producing a vacuum of 0.5 mm. The system was evacuated for about ten minutes at this vacuum, then closed, disconnected and kept for about a week. After that period the tubes were taken out, sealed immediately at the top and subjected to heating. The tubes containing lactic dehydrogenase, malic dehydrogenase and GOT were equilibrated for a week at 4°C. For lysozyme and ribonuclease A this was done at room temperature. For each of the enzyme system one tube containing the enzyme and the other containing the complex were sealed and heated. The other two tubes containing the enzyme and the complex respectively were not sealed. They were used as controls.

ACTIVITY MEASUREMENTS

After the heating, the tubes were taken out and solutions were prepared for the activity measurements.

(i) Lysozyme

Lysozyme, salt free, Worthington Biochemical Corporation, was used throughout. 0.13 mg/ml of lysozyme in distilled water was used for activity measurements. Also, for the complex, the amount of the enzyme used was 0.13 mg/ml. For both the heated lysozyme and the heated complex the concentration of the enzyme in solution was 0.7 mg/ml. Activity measurements were made according to the procedure of Shugar [1952]. A solution of *micrococcus lysodeikticus* (Worthington Biochemical Corporation) was prepared in M/15 phosphate buffer, pH = 7.1 (concentration 0.6 mg/ml). Both the enzyme and the substrate solutions were kept at 37°C for 10 minutes. 0.5 ml of the enzyme solution was mixed with 3 ml of the substrate solution at 37°C and the drop in absorbance per minute was monitored at a wavelength of 450 nm at 37°C on a Cary 14 spectrophotometer. The slope was calculated from the straight line and the activity was expressed as the rate of drop in the absorbance per minute per milligram of the enzyme in 3.5 ml of the assay volume.

(ii) Ribonuclease A

Sigma (Type IIA) enzyme was used. Activity measurements were made according to the method of Kalnitsky *et al.* [1959]. All the solutions were prepared such that one ml of the solution contained 0.1-0.2 mg of the enzyme. So a test-tube containing 2 ml of 0.1 M acetate buffer, pH 5.0 (μ = 0.065), 0.1 ml of the enzyme solution was added. To this was added one ml of 1% yeast ribonucleic acid solution in acetate buffer. The mixture was incubated for 4 minutes at 37°C at which time one ml of 0.75% uranyl acetate in 25% perchloric acid (72% W/W) was added. The mixture was cooled in an ice bath and

then centrifuged at 3°C. Aliquots of 0.1 ml of the supernatant liquid were diluted with 3 ml of water and the absorbance of the acid soluble oligonucleotides was measured on a Cary 14 spectrophotometer at 260 nm. The absorbance measurements were done at 37°C. Since RNA itself showed some absorbance under these conditions, a blank was performed and its absorbance value was substrated from the one which contained the enzyme. For each sample an average of three-five absorbancy readings were taken.

(iii) Lactic dehydrogenase

The activity measurements were by the method of Bergmeyer *et al.* [1963]. Lactic dehydrogenase (Boehringer Mannheim GmbH) was used. The enzyme solutions were prepared such that there was 0.02 mg/ml. 2.85 ml of phosphate-pyruvate solution (0.05 M phosphate buffer, pH 7.5; 3.1×10^{-4} M pyruvate) was taken. 0.015 ml of DPNH solution (ca. 8×10^{-3} M NADH, Boehringer Mannheim GmbH, in phosphate-pyruvate solution) was added. 0.1 ml of enzyme solution was added at 25°C and the drop in optical density was noted for every minute for 3 to 5 minutes at 25°C and at a wavelength of 340 nm. According to Wróblewski and La Due [1955] a unit (activity unit) is the amount of lactic dehydrogenase which changes the optical density of DPNH at 340 nm by 0.001 in the one minute in 3 ml of assay mixture and at 24-27°C. It follows that for 0.1 ml of the enzyme solution

$$(\Delta E_{340/min}) \times 1000 \times 10 = \text{LDH units/ml of enzyme solution}$$

(iv) Malic dehydrogenase

Malic dehydrogenase, Boehringer Mannheim GmbH, was used. The activity measurements were by the method of Bergmeyer and Bernt

[1963]. The enzyme solutions were prepared such that there was 0.01 mg/ml. The following solutions were taken in a cuvette.

2.75 ml of phosphate-aspartate solution (0.1 M phosphate buffer, pH 7.4; 4.2×10^{-2} M aspartic acid)

0.05 ml α-oxoglutaric acid (6×10^{-2} M neutralised with 0.1 M NaOH)

0.05 ml DPNH solution (1.2×10^{-2} M NADH in 1% sodium bicarbonate solution)

0.05 ml GOT suspension (Boehringer Mannheim GmbH, 1 mg/ml in 3 M ammonium sulphate solution). All these solutions were maintained at 25°C. To this, 0.1 ml of the enzyme solution was added. Immediately the drop in absorbance was noted for every minute for 3-5 minutes. These measurements were done at 25°C at a wavelength of 340 nm. The activity is defined as the amount of enzyme in one ml of the solution which changes the optical density of DPNH at 340 nm by 0.001 in one minute at 25°C with an assay volume of 3 ml. It follows that taking 0.1 ml of enzyme solution for assay

$(\Delta E_{340/min}) \times 1000 \times 10$ = MDH units/ml of enzyme solution

The spectrophotometric measurements were done on a Cary 14 spectrophotometer for all the enzyme systems. The lactic dehydrogenase, GOT and malic dehydrogenase enzyme samples as well as sodium pyruvate, NADH, and α-oxoglutaric acid samples were all gifts from Dr Jim Lindsay (personal communication).

The activity assay procedure employed for GOT was the same as that of malic dehydrogenase.

The activity values for the unheated enzyme and the enzyme-Ca-DPA complex were taken as standards for all the enzyme systems studied. The amounts of the enzyme in the tubes containing the

heated and the unheated enzyme and in those containing the heated and the unheated enzyme-Ca-DPA complex were the same. But the amounts of the enzyme in the tubes containing the enzyme and that in the tubes containing the enzyme-Ca-DPA complex were not the same for lactic dehydrogenase, malic dehydrogenase and GOT. All the four tubes involving lysozyme and ribonuclease A contained the same amounts of the enzyme. With both lysozyme and ribonuclease A the differences in the actual activity values observed between the unheated enzyme and the unheated enzyme-Ca-DPA complex may be due to slight inhomogenity in the latter case.

The absolute values of heat stabilisation should be treated with care since they are subjected to experimental conditions. The results have been presented to derive a qualitative picture of the involvement of Ca-DPA in imparting thermal stability to the enzymes like ribonuclease A and lactic dehydrogenase.

REFERENCES

Alderton, G. and Snell, N. (1963) *Biochem. Biophys. Res. Commun.* 10, 139.

Algie, J.E. (1980) *Curr. Microbiol.* 3, 387.

Allerhand, A., Cochran, D.W. and Doddrell, D. (1970) *Proc. Natl. Acad. Sci. U.S.A.* 67, 1093.

Allewell, N.M., Mitsui, Y. and Wyckoff, H.W. (1973) *J. Biol. Chem.* 248, 5291.

Alsaadi, B.M., Rossotti, F.J.C. and Williams, R.J.P. (1980a) *J. Chem. Soc. Dalton* 597.

Alsaadi, B.M., Rossotti, F.J.C. and Williams, R.J.P. (1980b) *J. Chem. Soc. Dalton* 813.

Anderson, D.G., Hammes, G.C. and Walz, Jr. F.G. (1968) *Biochemistry* 7, 1637.

Aue, W.P., Karhan, J. and Ernst, R.R. (1976) *J. Chem. Phys.* 64, 4226.

Baillie, E. and Murrell, W.G. (1974) *Biochim. Biophys. Acta.* 372, 23.

Bello, J., Iijima, H. and Kartha, A. (1979) *Int. J. Peptide Protein Res.* 14, 199.

Bergmeyer, H. and Bernt, E. (1963) In *Methods of Enzymatic Analysis* (Bergmeyer, H., ed.), p.757, Verlag Chemie, Academic Press, New York.

Bergmeyer, H., Bernt, E. and Hess, B. (1963) In *Methods of Enzymatic Analysis* (Bergmeyer, H., ed.), p.736, Verlag Chemie, Academic Press, New York.

Bigelow, J.H. (1946) In *Inorganic Synthesis* (Fernelius, W.C., ed.), Vol.II, p.203, McGraw-Hill Co., New York.

Black, S.H. and Gerhardt, P. (1962) *J. Bacteriol.* 83, 960.

Blackburn, P. and Jailkhani, B.L. (1979) *J. Biol. Chem.* 254, 12488.

Blackburn, P. and Gavilanes, J.G. (1980) *J. Biol. Chem.* 255, 10959.

Bloembergen, N. (1957) *J. Chem. Phys.* 27, 572.

Bolscher, B.G.J.M., Lenstra, J.A. and Kaptein, R. (1979) *J. Magn. Resn.* 35, 163.

Bradbury, J.H., Foster, J.R., Hammer, B., Lindsay, J. and Murrell, W.G. (1981) *Biochim. Biophys. Acta* 678, 157.

Bradbury, J.H., Howell, J.R., Johnson, R.N. and Warren, B. (1978) *Eur. J. Biochem.* 84, 503.

Bramley, R. and Johnson, R.N. (1971) *J. Chem. Soc. Chem. Commun.* 1309.

Brown, L.R. and Bradbury, J.H. (1975) *Eur. J. Biochem.* 54, 219.

Brown, L.R. and Bradbury, J.H. (1976) *Eur. J. Biochem.* 68, 227.

Brown, D.M., Dekker, C.A. and Todd, A.R. (1952) *J. Chem. Soc.* 2715.

Brown, D.M. and Todd, A.R. (1952) *J. Chem. Soc.* 52.

Brown, D.M. and Todd, A.R. (1953) *J. Chem. Soc.* 2040.

Bundi, A. and Wüthrich, K. (1979) *Biopolymers* 18, 285.

Campbell, I.D., Dobson, C.M., Williams, R.J.P. and Xavier, A.V. (1973a) *J. Magn. Resn.* 11, 172.

Campbell, I.D., Dobson, C.M., Williams, R.J.P. and Xavier, A.V. (1973b) *Ann. N.Y. Acad. Sci.* 222, 163.

Campbell, I.D., Dobson, C.M. and Williams, R.J.P. (1975a) *Proc. Royal Soc. Lond.* 345, 23.

Campbell, I.D., Dobson, C.M. and Williams, R.J.P. (1975b) *Proc. Royal Soc. Lond.* 345, 41.

Campbell, I.D., Lindskog, S. and White, A.I. (1974) *J. Mol. Biol.* 90, 469.

Campbell, I.D., Lindskog, S. and White, A.I. (1975) *J. Mol. Biol.* 98, 597.

Carlisle, C.H., Palmer, R.A., Mazumdar, S.K., Gorinsky, B.A. and Yeates, D.G.R. (1974) *J. Mol. Biol.* 85, 1.

Carty, R.P. and Hirs, C.H.W. (1968) *J. Biol. Chem.* 243, 5244.

Carty, R.P. and Hirs, C.H.W. (1968) *J. Biol. Chem.* 243, 5254.

Chow, S.T. and McAuliffe, C.A. (1975) *Progress in Inorganic Chemistry* 19, 51.

Chung, L., Rajan, K.S., Merdinger, E. and Grecz, N. (1971) *Biophys. J.* 11, 469.

Cotton, F.A. and Wilkinson, G. (1972) *Advanced Inorganic Chemistry*, Second Revised and Augmented Edition, Wiley Eastern Private Ltd., New Delhi.

Crestfield, A.M., Stein, W.H. and Moore, S. (1963) *J. Biol. Chem.* 238, 2413.

Davies, D.W. and Waind, G.M. (1950) *J. Chem. Soc.* 301.

Deakyne, C.A. and Allen, L.C. (1979) *J. Amer. Chem. Soc.* 101, 3951.

Dennis, E.A. and Westheimer, F.H. (1966) *J. Amer. Chem. Soc.* 88, 3431.

Dobson, C.M.and Levine, B.A. (1976) In *New Techniques in Biophysics and Cell Biology* (Pain, R.H. and Smith, B.E., eds), vol.3, p.19, Wiley, New York.

Dobson, C.M. and Williams, R.J.P. (1977) In *Metal-Ligand Interactions in Organic Chemistry and Biochemistry* (Pullman, B. and Goldbum, N., eds), Part I, p.255, Reidel.

Doi, R.H. and Halvorson, H. (1961) *J. Bact.* 81, 642.

Doscher, M.S. (1967) *Meth. Enzymol.* XI, 640.

Doscher, M.S. and Hirs, C.H.W. (1967) *Biochemistry* 6, 304.

Dwek, R.A. (1973) *Nuclear Magnetic Resonance (NMR) in Biochemistry, Applications to Enzyme Systems*, Clarendon Press, Oxford.

Dyson, G.M. (1961) *Org. Syn. Coll.* 1, 165.

Eckstein, F., Saenger, W. and Suck, D. (1972) *Biochem. Biophys. Res. Commun.* 46, 964.

Einsphar, H., Gartland, G.L. and Bugg, C.E. (1977) *Acta Cryst.* B33, 3385.

Ettinger, M.J. and Hirs, C.H.W. (1968) *Biochemistry* 7, 3374.

Figgis, B.N. and Lewis, J. (1964) In *Modern Co-ordination Chemistry* (Lewis, J. and Wilkins, R.G., eds), Chapter VI, p.406, Interscience Publishers Inc., New York.

Findlay, D., Herries, D.G., Mathias, A.P., Rabin, B.R. and Ross, C.A. (1962) *Biochem. J.* 85, 152.

Freedman, M.H., Cohen, J.S. and Chaiken, I.M. (1971) *Biochem. Biophys. Res. Commun.* 42, 1148.

Fusold, H., Turba, F. and Wirsching, W. (1961) *Biochem. Z.* 335, 86.

Galloway, G.J. (1978) Honours Thesis, Australian National University.

Gerhardt, P. and Murrell, W.G. (1978) In *Spores* VII (Chambliss, G. and Vary, J.C., eds), p.18, American Society for Microbiology, Washington D.C.

Glick, D.H. and Barnard, E.A. (1970) *Biochim. Biophys. Acta* 214, 326.

Glushko, V., Lawson, P.J. and Gurd, F.R.N. (1972) *J. Biol. Chem.* 247, 3176.

Goldfarb, A.R., Buchanan, D.N. and Sutton, D.E. (1974) *Bioorg. Chem.* 3, 260.

Gould, G.W. and Dring, G.J. (1975) *Nature* 258, 402.

Gould, G.W. and Hurst, A. (1969) *The Bacterial Spore*, Academic Press Inc. Ltd., London.

Grecz, N. and Smith, R. (1966) *Bacteriol. Proc.* 32.

Grenthe, J. (1961) *J. Amer. Chem. Soc.* 83, 360.

Gross, E. and Witkop, B. (1967) *Biochemistry* 6, 745.

Gutte, B. (1975) *J. Biol. Chem.* 250, 889.

Gutte, B. (1977) *J. Biol. Chem.* 252, 663.

Gutte, B. (1978) *J. Biol. Chem.* 253, 3837.

Haar, W., Maurer, W. and Rüterjans, H. (1974) *Eur. J. Biochem.* 44, 201.

Habeeb, F.S.A. (1960) *Can. J. Biochem.* 38, 493.

Hachisuka, Y., Tochikubo, K., Yokoi, Y. and Murachi, T. (1967) *J. Biochem. (Tokyo)* 61, 659.

Halvorson, H.O. (1953) *The Physiology of Bacterial Spore*, Technical University of Norwas, Trondheim, Norway.

Halvorson, H.O. (1957) *J. Appl. Bacteriol.* 20, 305.

Halvorson, H.O. and Howitt, C. (1961) In *Spores II* (Halvorson, H.O. ed.), p.149, Burgess Publishing Co., Minneapolis, Minn., U.S.A.

Hammes, G.G. (1968) *Advan. Protein Chem.* 23, 1.

Hashimoto, T., Black, S.H. and Gerhardt, P. (1960) *Can. J. Microbiol.* 6, 203.

Heinrikson, R.L. (1966) *J. Biol. Chem.* 241, 1393.

Hirs, C.H.W. (1962) *Brookhaven Symp. Biol.* 15, 154.

Hirs, C.H.W., Halmann, M. and Kycia, J.H. (1965) *Arch. Biochem. Biophys.* 111, 209.

Horseley, W.J., Sternlicht, H. and Cohen, J.S. (1970) *J. Amer. Chem. Soc.* 92, 680.

Iijima, H., Patrzyc, H. and Bello, J. (1977) *Biochim. Biophys. Acta* 491, 305.

Inagaki, F., Watanabe, K. and Miyazawa, T. (1979) *J. Biochem. (Japan)* 86, 591.

Inagaki, F. and Miyazawa, T. (1981) *Progress in Nuclear Magnetic Resonance Spectroscopy* 14, 67.

Inorganic Preparations (1981) Chemistry Department, Science, A.N.U.

James, T.L. (1975) *Nuclear Magnetic Resonance in Biochemistry, Principles and Applications*, Academic Press, New York.

Jentoft, J.E., Jentoft, N., Gerken, T.A. and Dearborn, D.G. (1979) *J. Biol. Chem.* 254, 4366.

Jentoft, J.E., Gerken, T.A., Jentoft, N. and Dearborn, D.G. (1981) *J. Biol. Chem.* 256, 231.

Johnson, L.F. and Jankowski, W.C. (1972) *Carbon-13 NMR Spectra: A Collection of Assigned, Coded and Indexed Spectra*, A Wiley-Interscience Publication, New York.

Kalnitsky, G., Hummel, J.P., Resnick, H., Carter, J.R., Barnett, L.B. and Dierks, C. (1959) *Ann. N.Y. Acad. Sci.* 81, 542.

Kartha, G., Bello, J. and Harker, D. (1967) *Nature* 213, 862.

King, N.L.R. and Bradbury, J.H. (1971) *Nature* 229, 404.

Klee, W.A. and Richards, F.M. (1957) *J. Biol. Chem.* 229, 489.

Koenigs, E., Jaeschke, W. and Kinne, G. (1921) *Ber. dtsch. Chem. Ges.* 54, 1351.

Koenigs, E., Kinne, G. and Weiss, W. (1924) *Ber. dtsch. Chem. Ges.* 57, 1172.

Kunitz, M. (1946) *J. Biol. Chem.* 164, 563.

Lenstra, J.A., Bolscher, B.G.J.M., Stob, S., Beintema, J.J. and Kaptein, R. (1979) *Eur. J. Biochem.* 98, 385.

Lewis, W.B., Jackson, J.A., Lemons, J.F. and Taube, H. (1962) *J. Chem. Phys.* 36, 694.

Lewis, J.C., Snell, N.S. and Bun, H.K. (1960) *Science* 132, 544.

Lindon, J.C. and Ferrige, A.G. (1980) *Progress in Nuclear Magnetic Resonance Spectroscopy* 14, 27.

Machuga, E. and Klapper, M.H. (1975) *J. Biol. Chem.* 250, 2319.

Machuga, E. and Klapper, M.H. (1977) *Biochim. Biophys. Acta* 481, 526.

Maeda, Y., Fujita, T., Sugiura, Y. and Kog, P.S. (1968) *J. Gen. Appl. Microbiol.* 14, 217.

Marinetti, T.D., Snyder, G.H. and Sykes, B.D. (1975) *J. Amer. Chem. Soc.* 97, 6562.

Marinetti, T.C., Snyder, G.H. and Sykes, B.D. (1976) *Biochemistry* 15, 4600.

Marinetti, T.D., Snyder, G.H. and Sykes, B.D. (1977) *Biochemistry* 16, 647.

Markham, R. and Smith, J.D. (1952) *Biochem. J.* 52, 558.

McConnell, H.M.M. and Robertson, R.E. (1958) *J. Chem. Phys.* 29, 1361.

Meadows, D.H. and Jardetzky, O. (1968) *Proc. Natl. Acad. Sci. U.S.A.* 61, 406.

Meadows, D.H., Roberts, G.C.K. and Jardetzky, O. (1969) *J. Mol. Biol.* 45, 491.

Means, G.E. and Feeney, R.E. (1968) *Biochemistry* 7, 2192.

McKay, A.F. (1952) *Chem. Revs.* 51, 301.

Morris, A.T. and Dwek, R.A. (1977) *Quart. Rev. Biophys.* 10, 421.

Mullaney, P.F. (1966) *Nature* 210, 953.

Murdock, A.L., Grist, K.L. and Hirs, C.H.W. (1966) *Arch. Biochem. Biophys.* 114, 375.

Murrell, W.G. (1961) *Sympos. Soc. Gen. Microbiol.* 11, 100.

Murrell, W.G. (1967) *Adv. Microbiol. Physiol.* 1, 133.

Murrell, W.G. (1969) In *The Bacterial Spore* (Gould, G.W. and Hurst, A., eds), Chapter 7, p.215, Academic Press Inc. Ltd., London.

Murrell, W.G. (1978) *Spore Newsletter* 6, 27.

Murrell, W.G. (1981) In *Sporulation and Germination, Proc. Eighth Inter. Spores Conf.* (Levinson, H.S., Sonenheim, A.L. and Tupper, D.J.,eds), American Society for Microbiology, Washington D.C., In Press.

Nagayama, K., Wüthrich, K., Bachmann, P. and Ernst, R.R. (1977) *Biochem. Biophys. Res. Commun.* 73, 99.

Nieboer, E. (1975) *Struct. Bonding* 22, 1.

Nitrogen Chemical Digest IV. The Chemistry of Guanidines (1950) p.17, American Cyanamid Co., New York, N.Y.

Paik, W.K. and Kim, S. (1972) *Biochemistry* 11, 2589.

Pasteur, L. (1861) *C.R. Acad. Sci.* 52, 16.

Perkins, S.J. and Wüthrich, K. (1978) *Biochim. Biophys. Acta* 536, 406.

Postmus, C., Magnusson, L.B. and Craig, C.A. (1966) *Inorg. Chem.* 5, 1154.

Powell, J.F. (1953) *Biochem. J.* 54, 210.

Rajan, K.S., Jaw, R. and Grecz, N. (1978) *Bioinorg. Chem.* 8, 477.

Reuban, J. (1975) Naturwissenschaften 62, 172.

Reuban, J. and Fiat, D. (1969) *J. Chem. Phys.* 51, 4909.

Richards, F.M. and Wyckoff, H.W. (1971) In *The Enzymes*, 3rd Edn. (Boyer, P.D., ed.), vol.4, p.647, Academic Press, New York.

Rieman, H. (1963) Ph.D. Thesis, Th. Bang., Copenhagen, Denmark,

Roberts, G.C.K., Dennis, E.A., Meadows, D.H., Cohen, J.S. and Jardetzky, O. (1969) *Proc. Natl. Acad. Sci. U.S.A.* 62, 1151.

Rode, L.J. and Foster, J.W. (1960) *J. Bact.* 79, 650.

Rüterjans, H. and Witzel, H. (1969) *Eur. J. Biochem.* 9, 118.

Shiner, R.L. and Neumann, F.W. (1944) *Chem. Revs.* 35, 351.

Shugar, D. (1952) *Biochem. Biophys. Acta* 8, 303.

Sillén, L.G. and Martell, A.E. (1964) *Stability Constants of Metal-Complexes:* Supplement No. 1, Special Publication No. 17, The Chemical Society, London.

Solomon, I. (1955) *Phys. Rev.* 99, 559.

Spackman, D.H., Stein, W.H. and Moore, S. (1958) *Anal. Chem.* 30, 1190.

Strahs, G. and Dickerson, R.E. (1965) *Spore Newsletter* 2, 30.

Strahs, G. and Dickerson, R.E. (1968) *Acta Cryst.* B24, 571.

Tang, T., Rajan, K.S. and Grecz, N. (1968) *Biophys. J.* 8, 1458.

Tichane, R.M. and Bennett, W.E. (1957) *J. Amer. Chem. Soc.* 79, 1293.

Usher, D.A. (1969) *Proc. Natl. Acad. Sci. U.S.A.* 62, 661.

Usher, D.A., Richardson, D.I. and Eckstein, F. (1970) *Nature* 228, 663.

Usher, D.A., Erenrich, E.S. and Eckstein, F. (1972) *Proc. Natl. Acad. Sci. U.S.A.* 69, 115.

Walker, E.J. (1977) Ph.D. Thesis, University of Sydney.

Walker, E.J., Ralston, G.B. and Darvey, I.G. (1975) *Biochem. J.* 147, 425.

Walker, E.J., Ralston, G.B. and Darvey, I.G. (1976) *Biochem. J.* 153, 329.

Walters, D.E. and Allerhand, A. (1980) *J. Biol. Chem.* 255, 6200.

Wang, J.H. (1968) *Science* 161, 328.

Warth, A.D. (1977) *Adv. Microbiol. Physiol.* 17, 1.

Warth, A.D. (1978) *J. Bacteriol.* 134, 699.

Warth, A.D. (1980) *J. Bacteriol.* 143, 27.

Watt, I.C. (1981) In *Sporulation and Germination: Proc. Eighth Intern. Spores Conf.* (Levinson, H.S., Sonenheim, A.L. and Tupper, D.J.,eds) American Society for Microbiology, Washington D.C., In Press.

Welling, G., Van den Berg, A., Caastra, W. and Beintema, J.J. (1973) *Proc. Int. Congr. Biochem. 9th*, 438.

Witzel, H. (1963) *Progr. Nucleic Acid Res.* 2, 221.

Wróblewski, F. and La Due, J.S. (1955) *Proc. Soc. Exp. Biol. Med.* 90, 210.

Wüthrich, K. (1976) *NMR In Biological Research: Peptides and Proteins*, North Holland Publishing Co., Amsterdam.

Wyckoff, H.W., Hardman, K.D., Allewell, N.M., Inagami, T., Johnson, L.N. and Richards, F.M. (1967) *J. Biol. Chem.* 242, 3984.

Young, I.E. (1959) *Can. J. Microbiol.* 5, 197.